REMARKS

Claims 1,2 and 4-20 were pending in the application. Claim 18 has been amended to correct for antecedent basis. Claims 21-23 have been added, support for which can be found throughout the application and, for example, on pages 82-83 (see, for example Table 1 and the accompanying text) .

No new matter has been added.

Rejections under 35 U.S.C. § 112

Claim 18 stands rejected under 35 U.S.C. § 112, second paragraph as allegedly indefinite for failing to particularly point out and distinctly claiming the subject matter which applicants regard as the invention.

Claim 18 has been amended to correct the antecedent basis, rendering this rejection moot.

In view of the foregoing, Applicants respectfully request that the rejection of claim 18 under 35 U.S.C. § 112, second paragraph be withdrawn.

Claims 15-20 stand rejected under 35 U.S.C. § 112, first paragraph because allegedly while being enabling for a method of inhibiting the expression of ribonuclease L in cell and tissue *in vitro* using antisense targeted to a nucleic acid encoding ribonuclease L, does not reasonably provide enablement for a method of inhibiting the expression of ribonuclease L *in vivo*, a method of treatment or a method of modulating RNAi using an antisense targeted to a nucleic acid encoding ribonuclease L. According to the Office:

> Claims 15-20 are drawn broadly to [the] inhibition of the expression of ribonuclease L in any cell *in vivo* for the treatment of any disease that is associated with ribonuclease L, including treating any disease or condition resulting from an infection, ...that is the result of aberrant apoptosis, or any cancer using antisense targeted to a nucleic acid encoding ribonuclease L...The specification does not demonstrate any correlation with the inhibition of ribonuclease L in cell culture and a treatment effect for any disease or condition associated with ribonuclease L.

(Office Action at page 4). Applicants respectfully disagree.

The present application teaches the skilled artisan how to make or use the claimed invention. The crux of the rejection set forth by the Office appears to be that the present application, and also the prior art, allegedly does not support a correlation of *in vitro* results with *in vivo* results, especially when there are no working examples in the present application to support *in vivo* claims. However, it should be noted that the absence of working examples "should never be the sole reason for rejecting the claimed invention on the grounds of lack of enablement," and "the specification need not contain an example if the invention is otherwise disclosed in such manner that one skilled in the art will be able to practice it without an undue amount of experimentation. *In re Borkowski*, 422 F.2d 904, 908, 164 USPQ 642, 645 (CCPA 1970))." (M.P.E.P § 2164.02)

In support of the Office's allegations that the field of antisense is unpredictable, the Office cited Agrawal *et al.* (Agrawal *et al.*, *Molecular Medicine Today*, Vol. 6, p. 72-81, February 2000, hereinafter the "Agrawal reference"), Branch (Branch, *TIBS*, 23:45-50, hereinafter, the "Branch reference"), Green *et al.* (Green *et al.* (Green *et al. J. Am Col.. Surg.*, Vol. 191, No. 1, July 2000, hereinafter the "Green reference"), and Jen *et al.* (Jen *et al.*, *Stem Cells*, 2000 Vol. 18:3078-19, hereinafter the "Jen reference"). However, the Office has failed to take into account numerous references which more accurately describe the status of the field of antisense technology at or prior to the filing date of the present application (September 12, 2001). When taken as a whole the state of the art, at the time of the application's filing, including the references cited by the Office, do *not* support the Office's allegation that the field of antisense is unpredictable and that there is no correlation between *in vitro* and *in vivo* results.

As a preliminary matter, the Office is reminded that whether the *in vivo* data is sufficient for a drug approval by the Food and Drug Administration is not the same as whether a correlation exists between *in vitro* and *in vivo* data to those of skill in the art for patentability purposes. (See, M.P.E.P. 2164.05, "considerations made by the FDA for approving clinical trials are *different* from those made by the PTO in determining whether a claim is enabled.", citations omitted, emphasis added).

According to the Office, the Jen reference discusses, "Often formulations and techniques for delivery *in vitro* (cell culture) are not applicable *in vivo* (whole organism)." (Office Action, page 6) Based on this reading of the Jen reference, the

Office alleges that the Jen reference indicates that the field of antisense is unpredictable. The Jen reference, however, does *not* teach that antisense therapy is wholly unpredictable and doomed to failure. For example, the Jen reference teaches:

> a number of phase I/II trials employing ONs [antisense oligonucleotides] have been reported, virtually all have been characterized by a lack of toxicity but only modest clinical effects. A recent paper by *Waters et al.* describing the use of a bcl-2-targeted ON in patients with non-Hodgkin's lymphoma is typical in this regard.

(Jen *et al.*, page 315, right column). The Jen reference reports potential problems with finding a *clinically* effective antisense drug, but does not state that antisense compounds cannot be used to modulate RNA-mediated interference or inhibit the expression of a gene *in vivo*.

According to the Office, the Green reference supports the allegation that antisense technology is unpredictable and would impose an undue burden upon a person of ordinary skill in the art. However, it appears that the Office is once again confusing the different standards between patentability and drug approval by the FDA, which as discussed above are not related nor dependent upon one another. Green states in fact, "Specific inhibition of gene expression using antisense ODNs has been demonstrated in numerous biologic systems," (Green *et al.*, page 95, right column). Green further states, that "many of the limitations to antisense studies outlined earlier have been relatively easily dealt with" and "*in vivo* delivery of [antisense oligonucleotides] appears to be *more efficient* than that which occurs *in vitro*." (Green *et al.*, page 97, left and right column, emphasis added). Furthermore, antisense compounds, "delivered intravenously or intraperitoneally to murine models of inflammation, organ transplantation, and tumor xenografts have shown *potent* antisense inhibition without the need for a delivery agent." (Green *et al.*, page 97, right column). Green also discusses the results of *in vitro* and *in vivo* experiments demonstrating the inhibition of Ha-Ras, c-Raf, PKC-α, bcl-2, and ICAM-1 expression to treat cancer, transplant rejection, and Crohn's disease in mice and/or humans. Although, Green states that the "evolution of antisense technology…is far from complete," when taken as a whole, the Green reference supports the concept that antisense technology is predictable and functional both *in vitro* and *in vivo*.

According to the Office, the Agrawal reference states:

> The cellular uptake of negatively charged oligonucleotides is one of the important factors in determining the efficacy of antisense oligonucleotides...in vitro, cellular uptake of antisense oligonucleotides depends on many factors, including cell type, kinetics of uptake, tissue culture conditions, and chemical nature, length and sequence of the oligonucleotide. Any one of these factors can influence the biological activity of an antisense oligonucleotide.

(Office Action, page 6). However, Agrawal does not say that these factors are insurmountable or would cause a person of ordinary skill in the art to perform undue experimentation. Rather, Agrawal reviews the factors that are involved in antisense chemistry, delivery, and design. The Agrawal reference does not say that antisense technology is unpredictable, and as discussed above in regard to the Green reference, demonstrates there has been great success using antisense to inhibit expression and treat diseases or conditions both *in vitro* and *in vivo*.

Although the Office cited the Branch reference as supporting its allegation of unpredictability, the Office has not cited any particular portion of the Branch reference that supports that antisense technology is unpredictable. Nonetheless, the Branch reference does not teach that antisense is unpredictable or that any technical difficulties are insurmountable. Rather, the Branch reference concludes by saying, among other statements, that "there is growing evidence that antisense molecules can be useful pharmacological tools when applied carefully," (Branch, page 50, left column) indicating that the skilled artisan sees promise in the use of antisense and although some experimentation may be required to refine the parameters, this experimentation would not be undue.

The Office also alleges that the references demonstrate that undue experimentation would be required to make and use the present invention *in vivo*. However, just because some experimentation may be required does not make it undue. "The test is not merely quantitative, since a considerable amount of experimentation is permissible, if it merely routine, or if the specification in question provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed." (M.P.E.P. § 2164.06). The present application provides a *reasonable amount* of guidance with respect to the direction in which the experimentation should proceed.

The present specification outlines the types of compounds that can be used and methods used to inhibit ribonuclease L expression in cells and tissues. The experiments that a person of ordinary skill in the art may have to undertake to use the invention do not make it undue. "The fact that experimentation may be complex does not necessarily make it undue, if the art typically engages in such experimentation." (M.P.E.P. § 2164.01). The types of experiments that the Office alleges are merely routine in the art, even if they are complex.

The Office also alleges that there is no correlation between *in vitro* and *in vivo* results. However, absolute certainty is not required. Instead correlation is dependent on the overall state of the prior art. The M.P.E.P sets forth that it is the overall the state of the art that is important for determining the unpredictability of a field, not one or two references. The M.P.E.P states:

> In this regard, the issue of 'correlation' is also dependent on the state of the prior art. In other words, if the art is such that a particular model is recognized as correlating to a specific condition, then it should be accepted as correlating unless the examiner has evidence that the model does not correlate. Even with such evidence, *the examiner must weigh the evidence for and against correlation and decide whether one skilled in the art would accept the model as reasonably correlating to the condition.* In *re Brana*, 51 F.3d 1560, 1566, 34 USPQ2d 1436, 1441 (Fed. Cir. 1995) (reversing the PTO decision based on finding that *in vitro* data did not support *in vivo* applications)...*A rigorous or an invariable exact correlation is not required* as stated in *Cross v. Iizuka*, 753 F.2d 1040, 1050, 224, USPQ 739, 747 (Fed. Cir. 1985).

(M.P.E.P 2164.02, emphasis added). It appears that the Office is under the impression that an "exact correlation" is required, while the M.P.E.P clearly states that it is not.

Contrary to the Office's allegations, there has been a great deal of success and correlation between *in vitro* and *in vivo* results in the field of antisense. In addition to the results reported upon in the Green reference, a recent survey of the scientific literature demonstrates that there is a correlation between *in vitro* results and *in vivo* data in the state of the art of the present invention and that it is not unpredictable. This survey demonstrates numerous examples of correlation between *in vitro* experiments and *in vivo* experiments. For example, in Smith *et al.* (*Clinical Cancer Research*, 7:400-406,

February 2001), data is discussed demonstrating inhibition of bcl-2 expression *in vitro* and *in vivo*. In O'Dwyer *et al.* (*Clinical Cancer Research*, 5: 3977-3982, December 1999), results are discussed where the administration of an antisense compound inhibited the expression of *c-raf*-1 mRNA *in vitro* and *in vivo*. Based on these results the authors performed a clinical trial in human patients where they also saw expression of *c-raf-1* inhibited. In Miyake *et al.*, (*Clinical Cancer Research*, 6:1655-1663, May 2000) the authors provide data that show the inhibition of TRPM-2 both *in vitro* and *in vivo*. In Berg *et al.*, (*The Journal of Pharmacology and Experimental Therapeutics*, 298:477-484, 2001) the authors demonstrate *in vitro* and *in vivo* inhibition of the expression of thymidylate synthase. In Tortora *et al.*, (*Clinical Cancer Research*, 7:2537-2544, August 2001) the authors discuss in the introduction previous results of where antisense oligonucleotide against protein kinase alpha type I (PKAI) inhibit expression *in vitro* and show antitumor activity *in vivo*. In Tortora the authors combine PKAI antisense compounds with bcl-2 antisense compounds and demonstrate *in vitro* inhibition along with anti-tumor activity *in vivo* characterized by reduced tumor volume and an increase survival, which is assumed to be due to the inhibition of PKAI and bcl-2. *Id.* Applicants attach hereto copies of the above-identified references along with other references that demonstrate a correlation between *in vitro* and *in vivo* data. These articles and others available in the art demonstrate that a person of ordinary skill in the art would accept that *in vitro* inhibition of a specific gene's expression *does* correlate with *in vivo* inhibition.

Therefore, the field of antisense is not unpredictable. A person of ordinary skill in the art would not believe the Office's assertion that there is no correlation between *in vitro* data and *in vivo* data. Thus, the specification enables the pending claims of the present application. Accordingly, Applicants respectfully request that the enablement rejection be withdrawn.

Rejections under 35 U.S.C. § 102/103

Claims 1, 2, and 11 stand rejected under 35 U.S.C. § 102 (b) or 35 U.S.C. § 103 as allegedly anticipated by or obvious over Ogawa et al. (WO 96/38034, hereinafter the "'034 reference"). According to the Office, the '034 reference discusses a 35-mer oligonucleotide that is complementary to SEQ ID NO:3 and that "since the prior art

oligonucleotides meet all the structural limitations of the claims, the prior art oligonucleotide would then be considered to 'inhibit expression' of the gene as claimed, absent evidence to the contrary." Applicants respectfully disagree.

The '034 reference discusses virus-resistant plant expressing 2',5'-oligoadenylic acid synthetase and ribonuclease L originating in animal cells and process for constructing the same. The '034 reference fails to teach or even suggest a compound 8 to 50 nucleobases in length targeted to a nucleic acid molecule encoding ribonuclease L (SEQ ID NO: 3), wherein the compound specifically hybridizes with the nucleic acid molecule encoding ribonuclease L protein and inhibits the expression of ribonuclease L. There is no extrinsic evidence or intrinsic evidence that the oligonucleotide disclosed in the '034 reference can inhibit the expression of ribonuclease L.

The Office cites MPEP § 2112 which deals with the subject of inherent activity in a composition when the prior art is silent as to the inherent function. However, just because a prior composition has some of the same characteristics as the present invention does not mean that it also contains all the features of the present invention, specifically the ability to inhibit the expression of ribonuclease L. As the MPEP states,

> The fact that a certain result or characteristic *may* occur or be present in the prior art *is not sufficient* to establish the inherency of that result or characteristic. *In re Rijckaert*, 9 F.3d 1531, 1534, 28 USPQ2d 1955, 1957 (Fed. Cir. 1993) (reversed rejection because inherency was based on what would result due to optimization of conditions, not what was necessarily present in the prior art); *In re Oelrich*, 666 F.2d 578, 581-82, 212 USPQ 323, 326 (CCPA 1981). *"To establish inherency, the extrinsic evidence 'must make clear that the missing descriptive matter is necessarily present in the thing described in the reference, and that it would be so recognized by persons of ordinary skill. Inherency, however, may not be established by probabilities or possibilities. The mere fact that a certain thing may result from a given set of circumstances is not sufficient.'"* *In re Robertson*, 169 F.3d 743, 745, 49 USPQ2d 1949, 1950-51 (Fed. Cir. 1999) (citations omitted)

(MPEP § 2112, emphasis added). The MPEP is clear that inherency is not something that may happen, or "be established by probabilities or possibilities" rather, "the examiner must provide a basis in fact and/or technical reasoning to reasonably support the determination that the allegedly inherent characteristic *necessarily* flows from the

teachings of the applied prior art." (MPEP § 2112)   In this instance, Applicants respectfully assert that the Office has failed to provide a basis in fact and/or technical reasoning to reasonably support that the prior art oligonucleotide inhibits the expression of ribonuclease L. Additionally the Office has failed to provide a basis in fact and/or technical reasoning that the prior art oligonucleotides inhibit the expression of ribonuclease by at least 60%, by 80% or more, or by 90% or more as new claims 21-23 recite.

The '034 reference also does not render the present invention obvious because there is no suggestion or motivation within the reference to inhibit the expression of ribonuclease L or to make a compound that inhibits the expression of ribonuclease L. It appears from the disclosure based on the words that are in English, such as "PCR" and specific vector names that the oligonucleotides were used to clone or sub-clone ribonuclease L. Without an English translation, however, Applicants cannot be certain. Based on this assumption, therefore, a person of ordinary skill in the art would not have been motivated to make a compound 8 to 50 nucleobases in length targeted to a nucleic acid molecule encoding ribonuclease L (SEQ ID NO: 3), wherein the compound specifically hybridizes with the nucleic acid molecule encoding ribonuclease L protein and inhibits the expression of ribonuclease L because there is no indication for what the compounds where used except possibly, PCR or cloning purposes.

Therefore, because the '034 reference does not teach or suggest every limitation of the claims, the present invention is not anticipated by or rendered obvious over the '034 reference. Accordingly, Applicants respectfully request that the rejections over the '034 reference be withdrawn.

Claims 1, 2, 11, 12, and 14 stand rejected under 35 U.S.C. § 102(b) or 35 U.S.C. § 103(a) as allegedly anticipated by or obvious over Ogawa *et al.* (U.S. Patent No. 6,320,099, hereinafter the "'099 reference").

As an initial matter, it appears that the present claims have been rejected under the wrong section of the U.S. code. The '099 reference was issued as a patent on November 20, 2001, which is after the filing date of the present application. Therefore, it appears that the present rejection should have been made under 35 U.S.C. § 102(e), not 35 U.S.C.

§ 102(b). Applicants assume that the rejection still would have been issued under 35 U.S.C. § 102(e) and hereby respond under this assumption. If Applicants are incorrect, Applicants respectfully request that the Office contact the undersigned to discuss the rejection.

According to the Office, the '099 reference discloses a 35-mer oligonucleotide that is complementary to SEQ ID NO:3. As with the '034 reference discussed above, the Office alleges that since the oligonucleotide possesses the structural features of the present invention it therefore inherently inhibits the expression of ribonuclease L. However, as the MPEP states, inherency requires a basis in fact and/or technical reasoning (see above), something the Office has not provided. The oligonucleotide discussed in the '099 reference is a primer that was used in PCR. There is no discussion of the oligonucleotide's ability to inhibit the expression of ribonuclease L and there is no implicit or explicit suggestion that the primer can be used to inhibit the expression of ribonuclease L. Furthermore, there is no evidence based on fact and/or technical reasoning that would demonstrate the oligonucleotide can inhibit the expression of ribonuclease L by at least 60%, by 80% or more, or by 90% or more. Therefore, the '099 references fails to anticipate the present invention.

Additionally, the '099 references fails to render the present invention obvious. The '099 reference does not discuss inhibiting the expression of ribonuclease L, rather the '099 reference discusses expressing ribonuclease L to produce viral resistant plants. Therefore, a person of ordinary skill in the art would not have been motivated to inhibit the expression of ribonuclease L based on a reference that intentionally increases the expression of ribonuclease L to increase virus resistance in plants. The presence of an oligonucleotide does not implicitly or explicitly suggest or motivate a person of ordinary skill in the art when the same oligonucleotide is used in a process that results in the expression of ribonuclease L in plants. Therefore, since the '099 reference discusses expressing ribonuclease L there would be no expectation of success that the primer could be used to inhibit the expression of ribonuclease L. It appears that the motivation used by the Office has been supplied by the Applicants' specification, which is strictly prohibited. Thus, the '099 reference fails to make the present invention obvious.

In view of the foregoing, Applicants respectfully request that the rejection of claims 1, 2, 11, 12, and 14 under 35 U.S.C. § 102 or 35 U.S.C. § 103 be withdrawn.

Claims 1, 2, and 4-15 stand rejected under 35 U.S.C. § 103(a) as allegedly unpatentable over Maitra *et al.* (*J. Virology*, Feb. 1998, pages 1146-1152) in view of Silverman *et al.* (U.S. Patent No. 6,028,243), Zhou *et al.* (*Cell*, 1993, Vol. 72: 753-765), Milner *et al.* (*Nature Biotechnology* 15:537-541, hereinafter the "Milner reference") and Baracchini *et al.* (U.S. Patent No. 5,801,154, hereinafter the "Baracchini reference")[1].

The Maitra reference discusses regulation of HIV replication by 2',5'-oligoadenylate-Dependent RNase L. The Maitra reference discusses introducing the RNase L gene in an antisense orientation by infecting cells with a virus containing the antisense oriented gene. However, the Maitra reference does not teach or even suggest using compounds that are smaller than the entire coding region, let alone compounds that are 8 to 50 nucleobases in length targeted to a nucleic acid molecule encoding ribonuclease L, wherein the compound specifically hybridizes with the nucleic acid molecule encoding ribonuclease L protein and inhibits the expression thereof. Based on what is discussed in the Maitra reference one of ordinary skill in the art would have no motivation to try compounds that are smaller than the coding region and there would be no expectation of success since there are no examples of compounds 8 to 50 nucleobases in length that inhibit the expression of ribonuclease L. Additionally, the Maitra reference fails to teach or even suggest compounds that have at least one modified linkage, at least one modified nucleobase, or at least one modified sugar moiety.

The Silverman reference discusses mice and cells with a homozygous disruption in the RNase L gene and methods thereof. However, the Silverman reference does not cure the deficiencies of the Maitra reference. The Silverman reference does not discuss inhibiting the expression of ribonuclease L by using antisense compounds that are 8 to 50 nucleobases in length targeted to a nucleic acid molecule encoding ribonuclease L, wherein the compound specifically hybridizes with the nucleic acid molecule encoding

---

[1] Applicants note that the exact reference for Milner and Barracchini were not included in the Office Action, see page 11, however, these references have been cited in similar cases so Applicants assume that these are the references for the present rejection. If Applicants are incorrect in this assumption, Applicants' undersigned representative requests to be notified immediately.

ribonuclease L protein. There is no suggestion to make and/or use such a compound. A person of ordinary skill in the art would not have been motivated to use the antisense compounds that are 8 to 50 nucleobases in length based because the gene disruption described in Silverman is based on homozygous disruption, a method that has no similarity to antisense technology.

The Zhou reference discusses the expression cloning of 2-5-A-Dependent RNAse, a uniquely regulated mediator of interferon action. However, the Zhou references fails to cure the deficiencies of the Maitra reference and the Silverman reference. The Zhou reference does not discuss or even suggest inhibiting the expression of ribonuclease L by using antisense compounds that are 8 to 50 nucleobases in length targeted to a nucleic acid molecule encoding ribonuclease L, wherein the compound specifically hybridizes with the nucleic acid molecule encoding ribonuclease L protein. A person of ordinary skill in the art would not have been motivated to use the antisense compounds that are 8 to 50 nucleobases in length based because the cloning of the gene does not suggest inhibiting the expression of the same gene using antisense compounds of the present invention.

According to the Office, the Milner reference discusses methods of screening for determining antisense compounds targeted to any known gene. However, the Milner reference does not cure the deficiencies of the Maitra, Silverman, and Zhou references. The Milner reference fails to teach or even suggest ribonuclease L. The Milner reference does not refer to the Maitra, Silverman, or Zhou references, and Milner fails to teach or even suggest a compound that is 8 to 50 nucleobases in length that specifically hybridizes to a nucleic acid molecule that encodes for ribonuclease L and inhibits the expression of ribonuclease L.

According to the Office, the Baracchini reference discusses the incorporation of phosphorothioate internucleotide linkages, 2'-O-methoxy ethyl sugar modification, 5 methyl cytosines and chimeric structures into antisense oligonucleotides to increase antisense stability and enhance affinity, as well as compositions comprising antisense oligonucleotides, pharmaceutically acceptable diluents and colloidal dispersion systems. However, the Baracchini reference does not cure the deficiencies of the Maitra, Silverman, Zhou, and Milner references. The Baracchini reference fails to teach or even

suggest ribonuclease L. The Baracchini reference does not refer to the other references, and Baracchini fails to teach or even suggest a compound that is 8 to 50 nucleobases in length that specifically hybridizes to a nucleic acid molecule that encodes for ribonuclease L and inhibits the expression of ribonuclease L.

The Office suggests that it would have been

> obvious to one of ordinary skill in the art to make an antisense molecule targeted to ribonuclease L because Maitra et al. teach that antisense is a viable means to inhibit the expression of ribonuclease L in cells in vitro and Silverman et al. teach that a cell line comprising an inhibition of ribonuclease L was useful as a tool to screen for antiviral drugs, in vitro. It would have been obvious to one of ordinary skill in the art to make an antisense oligonucleotide targeted to a nucleic acid encoding ribonuclease L, as taught by Maitra et al. of a length within the range of 8-50 nucleobases...because antisense of a short length are more easily synthesized and easier to deliver to cell than a vector expressing a full length antisense and because this length was convention in the art.

(Office Action, page 13). Furthermore, The Office alleges that one skilled in the art would have been motivated to make an antisense compound targeted to a nucleic acid encoding ribonuclease L because Silverman allegedly teaches inhibiting the expression of ribonuclease L for antiviral drug screening and antisense was a well known means for inhibiting the expression of a target molecule in vitro and Maitra allegedly teaches antisense that inhibits ribonuclease L. Applicants respectfully disagree.

In establishing a *prima facie* case of obviousness under 35 U.S.C. §103, it is incumbent upon the Examiner to provide a reason why one of ordinary skill in the art would have been led to modify a prior art reference or to combine reference teachings to arrive at the claimed invention. *Ex parte Clapp*, 227 U.S.P.Q. 972 (Bd. Pat. App. Int. 1985). To this end, the requisite motivation must stem from some teaching, suggestion or inference in the prior art as a whole or from the knowledge generally available to one of ordinary skill in the art and not from appellants' disclosure, see for example, *Uniroyal Inc. v. Rudkin-Wiley Corp.*, 5 U.S.P.Q.2d 1434 (Fed. Cir. 1988); and *Ex parte Nesbit*, 25 U.S.P.Q.2d 1817, 1819 (Bd. Pat. App. Int. 1992). In this respect, the following quotation

from *Ex parte Levengood*, 28 U.S.P.Q.2d 1300, 1302 (Pat. Off. Bd. App. 1993), is noteworthy:

> Our reviewing courts have often advised the Patent and Trademark Office that it can satisfy the burden of establishing a *prima facie* case of obviousness only by showing some objective teaching in either the prior art, or knowledge generally available to one of ordinary skill in the art, that "would lead" that individual "to combine the relevant teachings of the references." ... Accordingly, an examiner cannot establish obviousness by locating references which describe various aspects of a patent Applicants' invention without also providing evidence of the motivating force that would **impel** one skilled in the art to do what the patent Applicants have done. (citations omitted; emphasis added)

Significantly, the Office Action identifies no "motivating force" that would "impel" persons of ordinary skill to modify the respective teachings of the cited references and achieve the claimed invention. Additionally, the Office has failed to identify the motivation to "impel" the skilled artisan to combine the cited references.

Rather, the Office Action makes a general statement that it would be obvious to combine teachings of a reference discussing a particular gene with a reference that discusses antisense. Such a generalized motivation **is not** a "motivating force" that would "impel" persons of ordinary skill to modify the respective teachings of the cited references and achieve the claimed invention. Such a statement, at most, raises an inappropriate "obvious to try" standard. Indeed, the court made it clear that it is improper to reject claims as "obvious to try" where the motivation to combine references arises merely because the subject matter of the claimed invention is a promising field for experimentation, although the prior art provides only general guidance as to particular form of the claimed invention or how to achieve it. *In re O'Farrell*, 7 U.S.P.Q.2d 1673, 1681 (Fed. Cir. 1988). Without more specific suggestions in the prior art, there is insufficient motivation to combine the cited references. Furthermore, "focusing on the obviousness of substitutions and differences, instead of the invention as a whole, is a legally improper way to simplify the often difficult determination of obviousness." *Gillette Co. v. S.C. Johnson & Son*, 16 U.S.P.Q.2d 1923, 1927 (Fed. Cir. 1990).

Furthermore, it appears that the Office is using the disclosure of Silverman as a basis of motivation to inhibit ribonuclease L with antisense compounds because of Silverman's alleged discussing "inhibiting the expression of ribonuclease L for antiviral drug screening." (Office action, page 14). However, this motivation is not what Silverman provides. Rather, Silverman states, "Mutant non-human mammals comprising a homozygous disruption in the RNase L gene are useful for screening for drugs that *induce* transcription of RNase L or activate RNase L." (Silverman *et al.*, Col. 4, lines 27-30). Therefore, Silverman, would at most, motivate one to disrupt the gene via homozygous disruption to make a mutant animal or cell line that does not express RNase L and to screen for factors that can activate RNase L or increase the transcription of RNase L. The disruption methodology is different from antisense methodology and it would be difficult, if not impossible to find activators of RNase L transcription using antisense since the goal of antisense is to inhibit transcription and expression. Therefore, one skilled in the art would not have been motivated to use antisense by the teachings of Silverman even in view of the teachings of Maitra because Silverman teaches a technique that is completely different.

In addition, it appears that the only motivation that the Office is using to combine the references is the use of the Applicants' specification and hindsight reconstruction, which is strictly forbidden. *In re Fine*, 5 U.S.P.Q.2d 1596 (Fed. Cir. 1988) ("One cannot use hindsight reconstruction to pick and choose among isolated disclosures in the prior art to deprecate the claimed invention."). When assessing whether or not a combination of references would have produced a claimed invention, one must consider the teaching of each reference as a whole without undue emphasis on those features that would support a finding of obviousness. *In re Wesslau*, 147 U.S.P.Q. 391 (C.C.P.A. 1965) (it is impermissible to pick and choose from any one reference only so much of it as will support a given position to the exclusion of other parts necessary to the full appreciation of what the references fairly suggest to one of ordinary skill in the art).

Consideration of the cited references as a whole for what they each fairly suggest, demonstrates that a person of ordinary skill seeking to combine them would not have produced any claimed invention. In this respect, the Office Action has apparently picked one particular element from the Maitra reference, one particular element from the

Silverman reference, one particular element from the Zhou reference, one particular element from the Milner reference, and/or one particular element from the Barrachini reference. One skilled in the art, however, would *not* be motivated to pick and choose only those specific elements referred to in the Office Action from the many elements recited in the references and combine the selected elements in the specific manner indicated in the Office Action. Indeed, it appears that the only guide to picking and choosing particular elements from the cited art of records appears to have been the present application. Thus, the combination of references is improper for, at the very least, failure to provide motivation to combine references and for its use of hindsight reconstruction based upon Applicants' disclosure.

The Federal Circuit has recently affirmed the requirement for motivation to combine references, stating that:

> virtually all [inventions] are combinations of old elements. Therefore, an examiner may often find every element of a claimed invention in the prior art. If identification of each claimed element in the prior art were sufficient to negate patentability, very few patents would ever issue. Furthermore, rejecting patents solely by finding prior art corollaries for the claimed [**10] elements would permit an examiner to use the claimed invention itself as a blueprint for piecing together elements in the prior art to defeat the patentability of the claimed invention . . .

> To prevent the use of hindsight based on the invention to defeat patentability of the invention, this court requires the examiner to show a motivation to combine the references that create the case of obviousness. In other words, the examiner must show reasons that the skilled artisan, confronted with the same problems as the inventor and *with no knowledge of the claimed invention*, would select the elements from the cited prior art references for combination in the manner claimed . . .

> To counter this potential weakness in the obviousness construct, the suggestion to combine requirement stands as a critical safeguard against hindsight analysis and rote application of the legal test for obviousness.

Yamanouchi Pharm. Co. v. Danbury Pharm, Inc., 231 F.3d 1339 (Fed. Cir. 2000); 56 U.S.P.Q.2D 1641, 1645, citing *In re Rouffet*, 149 F.3d 1350, 1357-58, 47 USPQ2d 1453, 1457-8 (Fed. Cir. 1998) (emphasis supplied).

It appears that the Office has done what Yamanouchi reaffirms should not be done -- used Applicants' specification as a blueprint.

Thus, in view of the foregoing, Applicants respectfully submit that the Office has failed to establish a *prima facie* case of obviousness. In particular, the Office has failed to provide any motivation that would **impel** one skilled in the art to modify the cited references so as to produce Applicants' claimed inventions. Indeed, none of the references cited in the Office Action, nor the combinations thereof, suggest using compounds that are 5 to 50 nucleobases in length specifically hybridize to a polynucleotide that encodes for ribonuclease L. Further, none of the references cited in the Office Action suggest using compounds 8 to 50 nucleobases in length that inhibit the expression of ribonuclease L. Thus, none of the references cited by the Examiner suggest modifying or combining any of the cited references so as to arrive at the claimed invention.

Accordingly, Applicants respectfully request the rejection under 35 U.S.C. § 103(a) be withdrawn.

Conclusion

Applicants believes the claims are in condition for allowance. An early Notice of Allowance is therefore earnestly solicited. Applicants invite the Examiner to contact the undersigned at (215) 665-6928 to clarify any unresolved issues raised by this response.

Respectfully submitted,

Daniel M. Scolnick, Ph.D.
Reg. No. 52,201

COZEN O'CONNOR, P.C.
1900 Market Street
Philadelphia, PA 19103-3508
Telephone: (215) 665-2000
Facsimile: (215) 701-2029

Attachments:
Smith *et al. Clinical Cancer Research*, 2001, 7, 400-406
O'Dwyer *et al. Clinical Cancer Research*, 1999, 5, 3977-3982
Miyake *et al. Clinical Cancer Research*, 2000, 6, 1655-1663
Berg *et al. J. Pharmacology and Experimental Therapeutics*, 2001, 298, 477-484
Tortora *et al. Clinical Cancer Research*, 2001, 7, 2537-2544

Combined Blockade of Protein Kinase A and Bcl-2 by Antisense Strategy Induces Apoptosis and Inhibits Tumor Growth and Angiogenesis[1]

Giampaolo Tortora,[2] Rosa Caputo,
Vincenzo Damiano, Roberto Bianco,
Gabriella Fontanini, Sabina Cuccato,
Sabino De Placido, A. Raffaele Bianco, and
Fortunato Ciardiello

Cattedra di Oncologia Medica, Dipartimento di Endocrinologia e
Oncologia Molecolare e Clinica, Università Federico II, 80131 Napoli
[G. T., R. C., V. D., R. B., S. D. P., A. R. B., F. C.], and Istituto di
Anatomia Patologica, Dipartimento di Oncologia, Università di Pisa,
56100 Pisa [G. F., S. C.], Italy

ABSTRACT

Protein kinase A type I (PKAI) plays a key role in neoplastic transformation, conveys mitogenic signals from different sources, and is overexpressed in the majority of human tumors. Inhibition of PKAI by different tools results in cancer-cell growth inhibition *in vitro* and *in vivo*. We and others have recently shown that a novel class of mixed-backbone oligonucleotides targeting the PKAI subunit RIα exhibits improved pharmacokinetic properties and antitumor activity accompanied by increased apoptosis in several human cancer types *in vitro* and *in vivo*. The role of bcl-2 in the control of apoptosis has been widely documented, and the inhibition of bcl-2 expression and function may have important therapeutic implications. In fact, oligonucleotides antisense bcl-2 have shown antitumor activity in animal models and have successfully completed early clinical trials. Recent studies have demonstrated a direct role of PKA in the regulation of the bcl-2-dependent apoptotic pathway. Therefore, we have investigated the combined blockade of PKA and bcl-2 by antisense strategy as a potential therapeutic approach. The novel hybrid DNA/RNA mixed-backbone oligonucleotide antisense RIα (AS RIα) in combination with the antisense bcl-2 (AS bcl-2), cooperatively inhibited bcl-2 expression and soft agar growth and induced apoptosis in different human cancer cell lines. p.o. administration of

AS RIα in combination with i.p. AS bcl-2 caused a marked antitumor effect and a significant prolongation of survival in nude mice bearing human colon cancer xenografts. Moreover, histochemical analysis of tumor specimens showed inhibition of RIα and Ki67 expression, inhibition of angiogenesis, and parallel induction of apoptosis *in vivo*. The results of our study imply an interaction between the PKA and bcl-2 signaling pathways and, because both antisenses have now entered Phase II trials, provide the rationale to translate this novel therapeutic strategy in a clinical setting.

INTRODUCTION

PKA[3] plays a key role in the control of cell growth and differentiation of mammalian cells. PKA is composed of two distinct isoforms, PKAI and PKAII, which differ only in their regulatory subunits, defined RI and RII, respectively (1). PKAI is involved in cell proliferation and neoplastic transformation, is required for the G_1-S transition of cell cycle, and mediates mitogenic signals from different growth factors, including transforming growth factor α and epidermal growth factor (1–3). PKAI overexpression is detected in the majority of human cancers, correlating with worse clinicopathological features in colon, breast, and ovarian cancer (2). Conversely, PKAII is preferentially expressed in normal tissues and is involved in growth arrest, differentiation, and induction of apoptosis (1). PKAI subunit RIα has a structural interaction with the ligand-activated epidermal growth factor receptor as well with other receptors, cooperating in the propagation of mitogenic signals originated by different growth factors and hormones (2). For the above reasons, PKAI represents a relevant target for therapeutic intervention.

Down-regulation of PKAI by different pharmacological tools, including AS oligodeoxynucleotides targeting its RIα subunit, causes cell growth arrest and differentiation in a variety of cancer cell lines (2, 4) and shows antitumor activity in nude mice (5). Recently, these effects have been obtained also with a novel class of oligonucleotides containing MBOs. MBOs exhibit improved pharmacokinetic and toxicological properties *in vivo* as compared with PS oligonucleotides (6). We and others have shown the antitumor effect of a MBO AS RIα and its synergism with different classes of cytotoxic drugs in several human cancer xenografts in nude mice (2, 7). Recently, a hybrid DNA/RNA MBO AS RIα (defined as GEM 231) has completed a Phase I clinical trial in cancer patients with negligible toxicity

(8). We and others have recently shown that the novel AS RIα exhibits a good pharmacokinetic profile and inhibits tumor growth in cooperation with several anticancer drugs with p.o. administration (9, 10).

Bcl-2 is the prominent member of a family of proteins responsible for dysregulation of apoptosis, prevention of cancer-cell death, and resistance to chemotherapy and radiotherapy (11, 12). Antiapoptotic bcl-2 family members counteract proapoptotic proteins, such as BAD and BAX, in the control of the pathways leading to the release of cytochrome c from the mitochondrial membrane, to the activation of the caspases cascade, and, finally, to the execution of apoptosis (11–13). Paclitaxel and other microtubule-damaging agents cause apoptosis, inducing bcl-2 phosphorylation and inactivation (12, 14). These effects may be obtained also by noncytotoxic tools, including AS bcl-2 oligonucleotides. A 18-mer PS- oligonucleotide targeting the human bcl-2 (G3139; GENTA) is able to block bcl-2 expression, inhibit the growth of human melanoma and prostate cancer in animal models, and synergize with cytotoxic drugs (15, 16). Moreover, it is able to delay the progression to androgen-independence (16). This same effect was obtained in a Shionogi murine tumor by an oligonucleotide targeting the murine bcl-2 (17). The AS human bcl-2 has completed early clinical studies in different malignancies including melanoma, non-Hodgkin's lymphoma, and prostate cancer, demonstrating antitumor activity alone and in combination with cytotoxic drugs (18–20).

In past years, a large number of studies elucidated the role of different signaling proteins in the control of bcl-2-dependent apoptotic events. In addition to JNK and Akt proteins (11, 12, 21, 22), PKA seems to play a prominent role. In fact, after treatment with paclitaxel and other microtubule-damaging agents, PKA as well as JNK cause specific phosphorylation of bcl-2 and activate the cascade that leads to apoptosis (23, 24). Agents that inhibit PKAI and induce PKAII, such as AS RIα or DBcAMP, are able to inhibit bcl-2 expression and function, to induce cleavage of PARP and caspase 3 activation, causing apoptosis (25, 26). Taken together, these data suggest that the combined blockade of PKAI and bcl-2 may have a therapeutic potential. Therefore, in the present study, we have used two AS oligonucleotides as selective tools. We have investigated whether the hybrid MBO AS RIα and the PS-AS bcl-2, used in combination, have any cooperative effect on tumor growth and apoptosis of different human cancer cells in vitro and in vivo. Moreover, we have performed immunohistochemical analysis of tumor specimens to evaluate their in vivo effect on cell proliferation, angiogenesis, and apoptosis.

MATERIALS AND METHODS

Oligodeoxynucleotides. All oligonucleotides were synthesized by Dr. Sudhir Agrawal at Hybridon, Inc. (Milford, MA). The PS-AS bcl-2, corresponding to the first six codons of bcl-2 mRNA site and its mismatch control oligonucleotide have the following sequences: TCTCCCAGCGTGCGCCAT and TCTCCCAGCATGTGCCAT, respectively, as published previously (15). The AS RIα MBO is a hybrid oligonucleotide targeted against the NH$_2$-terminal 8–13 codons of the RIα regulatory subunit of PKA (5) with the following sequence:

GCGUGCCTCCTCACUGGC; the RIα control oligonucleotide is a scramble MBO obtained by mixing all four nucleosides at each position (10). The two MBO oligonucleotides contain PS internucleotide linkages, identified by roman type for the nucleosides flanking each position, and 2'-O-methyl-ribonucleosides modifications, identified by italic type. Oligonucleotides were synthesized, identified, and purified according to the protocol described earlier (27).

Cell Growth Experiments and AS Treatment. GEO human colon cancer cells were grown in McCoy medium, OVCAR-3 human ovarian and ZR-75-1 human breast cancer cells were maintained in DMEM (Flow Laboratories, Irvine, Scotland) supplemented with 10% heat-inactivated fetal bovine serum, 20 mM HEPES, (pH 7.4), penicillin (100 IU/ml), streptomycin (100 μg/ml), and 4 mM glutamine (ICN, Irvine, United Kingdom) in a humidified atmosphere of 95% air and 5% CO$_2$ at 37°C. For cell growth experiments in soft agar, 10^4 cells/well were seeded in 24 multiwell cluster dishes as described previously (28) and treated with different concentrations of the indicated oligonucleotides. In all of the combination experiments shown, the two oligonucleotides were administered simultaneously, (AS bcl-2 on days 1–3, AS RI MBO or Scramble MBO on days 1 and 3). We also performed experiments by administering the compounds in a sequential fashion, using the AS bcl-2 on days 1, 2, and 3 with the MBOs on days 4 and 6 or, alternatively, the MBOs on days 1 and 3 and the AS bcl-2 on days 4, 5, and 6. Twelve days after the last treatment, cells were stained with nitroblue tetrazolium (Sigma Chemical Co., Milan, Italy), and colonies >0.05 mm were counted.

Western Blot Analysis. Total cell lysates (50 μg) were fractionated through 7.5% or 12% SDS-polyacrylamide gels, transferred to nitrocellulose filters, incubated with specific MAbs and then horseradish-peroxidase antiserum (Bio-Rad Laboratories, Milano, Italy). Immunoreactive proteins were visualized by enhanced chemiluminescence (Amersham International, United Kingdom), as described previously (3). Antihuman RIα mouse MAb (Transduction Laboratories, Lexington, KY), antihuman bcl-2 mouse MAb (Santa Cruz Biotechnology, Santa Cruz, CA), and antihuman actin mouse MAb (Sigma Chemical Co.) were used following the methods described previously (3).

Apoptosis in Cultured Cells. The induction of apoptosis was determined by the Cell Death Detection ELISA Plus Kit, which detects cytosolic histone-associated DNA fragments (Roche Molecular Biochemicals, Mannheim, Germany). Briefly, GEO and OVCAR-3 cells (5 × 10^4 cells/dish) were seeded into 35-mm dishes. After treatment with different concentrations of either AS RI (days 1 and 3), Scramble MBO (days 1 and 3, or AS bcl-2 (days 1, 2, and 3), alone and in combination, on day 4 cells were washed once with PBS then 0.5 ml of lysis buffer was added. After a 30-min incubation, the supernatant was recovered and assayed for DNA fragments as recommended by the manufacturer. Each treatment was performed in quadruplicate. Total number of cells was measured with an hemocytometer in additional plates receiving an identical treatment. The values resulting from the readings of optical density at 405 nm ($A_{280 nm}$) were normalized for cell number, and the ratio of optical density-treated cells:optical density-untreated cells was defined as the apoptotic index.

GEO Xenografts in Nude Mice. Female Balb/cAnNCrlBR athymic (nu+/nu+) mice 5–6 weeks of age were purchased from Charles River Laboratories, Milan, Italy. The research protocol was approved and mice were maintained in accordance with institutional guidelines of the University of Naples Animal Care and Use Committee. Mice were acclimated to the University of Naples Medical School Animal Facility for 1 week before being injected with cancer cells. GEO cells were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum, as described above. Then, 10^7 cells were resuspended in 200 μl of Matrigel (Collaborative Biomedical Products, Bedford, MA) and injected s.c. in mice (7, 29). After 7 days, when well established tumors of approximately 0.2 cm³ were detected, mice were randomized to receive different treatments. Ten mice for each group were treated with either p.o. AS RIα (10 mg/kg/dose) in a 5% dextrose solution, i.p. AS bcl-2 (10 mg/kg/dose), or i.p. mismatch control bcl-2 (10 mg/kg/dose) alone or in combination (AS RIα + AS bcl-2 or AS RIα + mismatch bcl-2) for 5 days a week for a total of 3 weeks. Tumor volume was measured using the formula: $\pi/6 \times$ larger diameter $\times$ (smaller diameter)^2, as previously reported (29).

Immunohistochemical Analysis. Formalin-fixed, paraffin-embedded tissue sections (5 μm) were processed as reported previously (30). Reactions with appropriate primary antibody, secondary biotinylated goat antibody (1:200 dilution; Vectastain ABC kit; Vector Laboratory, Burlingame, CA), avidin-biotinylated horseradish peroxidase H complex, diaminobenzidine, and hydrogen peroxide and counterstaining with hematoxilin were as described previously (30).

The following antibodies were used in this study: an anti-RIα MAb (Transduction Laboratories, Lexington, KY), 1:100 dilution; and an anti-Ki67 MAb (clone MIB1; DBA, Milan, Italy), 1:100 dilution. All analyses were performed in a blind fashion. To determine the percentage of positive cells, at least 1000 cancer cells/slide were counted and scored at X40.

New blood vessels were detected as described previously (31) using a MAb against the human factor VIII-related antigen (Dako, Milan) at a dilution of 1:50 and stained using a standard immunoperoxidase method (Vectastain ABC kit; Vector). Each slide was first scanned at low power (X10–100), and the area with the higher number of new vessels was identified (the "hot spot"). This region was then scanned at X250 (0.37 mm²). Stained blood vessels were counted in each of five different fields. For individual tumors, microvessel count was scored by averaging the five fields counts.

Determination of apoptosis was performed by a modified TdT-mediated nick end labeling assay (32) with the ApopTag Kit (Intergen, DBA, Milan, Italy). The processing of paraffin-embedded tissues, washings, the addition of working-strength TdT enzyme, antidigoxigenin peroxidase conjugate, and diaminobenzidine and counterstaining with hematoxilin were carried out according to the ApopTag Kit manual and as described previously (32). The counting of apoptotic nuclei was performed at X40, evaluating at least 100 cells and an average of 10 fields.

Statistical Analysis. The Mantel-Cox log-rank test (33) was used to evaluate the statistical significance of the results. All reported Ps were two-sided. All analyses were performed with the BMDP New System statistical package

version 1.0 for Microsoft Windows (BMDP Statistical Software, Los Angeles, CA).

Table 1 IC$_{50}$ (μM) of different oligonucleotides

The effect of the different oligonucleotides was measured on the soft agar growth of GEO, ZR-75-1, and OVCAR-3 human cancer cells (described in "Materials and Methods"). The data represent the means of triplicate determination of at least two experiments with SDs < 15%.

	AS RI MBO	Scramble MBO	AS bcl-2	Bcl-2 control
GEO	0.8	>25	4.6	>15
ZR-75-1	1.2	>25	3	>15
OVCAR-3	0.7	>25	7	>15

RESULTS

Effect of Different Oligonucleotides on the Growth of Human Cancer Cells. We have evaluated the antitumor activity of the MBO AS RIα, its control scramble MBO, the PS-AS bcl-2, and its mismatch control on the soft agar growth of a variety of human cancer cells, including GEO colon, OVCAR-3 ovarian, and ZR-75-1 breast cells. As shown in Table 1, the AS RIα caused a dose-dependent growth inhibition on all tested cells, achieving the IC$_{50}$ at doses ranging between 0.7 and 1.2 μM. Conversely, an IC$_{50}$ could not be reached with the control scramble MBO, even with doses as high as 25 μM. A dose-dependent growth inhibitory effect was also observed in all cell lines with the AS bcl-2, with an IC$_{50}$ ranging between 3 and 7 μM, whereas the same degree of inhibition was reached only by higher concentrations of mismatch bcl-2 oligonucleotide (Table 1).

Cooperative Effect of AS RIα and AS Bcl-2. We evaluated whether the AS RIα and AS bcl-2 in combination were able to cooperate in inhibiting human cancer cell growth. We selected different doses of AS RIα (0.1–1 μM), Scramble MBO (0.5 and 1 μM), AS bcl-2 (0.5–5 μM), and mismatch bcl-2 (1–5 μM) and used these oligonucleotides alone or in combination. Different schedules of treatment were studied, administering the two oligonucleotides simultaneously and, in a sequential order, giving either the AS bcl-2 first and then the AS RIα or *vice versa*. Remarkably, the antiproliferative effect obtained was similar, regardless of the schedule used (data not shown). For this reason, thereafter we performed all of the experiments using a simultaneous administration, as described in "Materials and Methods." Fig. 1 shows that AS RIα and AS bcl-2, when used in combination, have a dose-dependent cooperative effect, which is particularly evident with lower doses, on GEO, ZR-75-1, and OVCAR-3 soft agar colony formation. For instance, in ZR-75-1 breast cancer cells, AS RIα (0.1 μM) and AS bcl-2 (2 μM), which alone cause 10% and <40% inhibition, respectively, completely suppressed colony formation in soft agar (Fig. 1, *middle*). Conversely, the combination of Scramble MBO (0.5 μM) with increasing doses of AS bcl-2 caused, at most, an additive effect (Fig. 1). In the same fashion, the addition of mismatch bcl-2 oligonucleotide to AS RIα MBO did not modify the inhibitory effect obtained with the latter alone in GEO cells (data not shown).

Inhibition of Bcl-2 Expression. We measured the effect of the AS oligonucleotides on the expression of the target gene
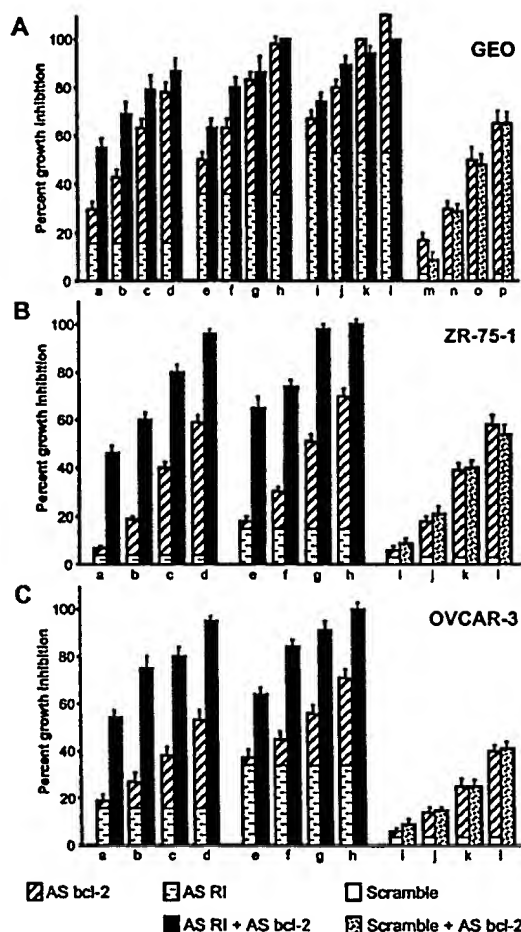
Fig. 1 Effect of the AS RIα MBO, scramble MBO and AS bcl-2 on GEO, ZR-75-1, and OVCAR-3 cell growth in soft agar. Concentrations of AS bcl-2 were 0.5, 1, 2, and 5 μM in all cell lines. *A*, in GEO cells, AS RIα doses are: 0.1 μM (*a–d*); 0.5 μM (*e–h*); and 1 μM (*i–l*). AS bcl-2 doses are 0.5 μM (*a, e, i,* and *m*); 1 μM (*b, f, j,* and *n*); 2 μM (*c, g, k,* and *o*); and 5 μM (*d, h, l,* and *p*) in all cell lines. In ZR-75-1 (*B*) cells and OVCAR-3 (*C*) cells, AS RIα doses are 0.1 μM (*a–d*) and 0.5 μM (*e–h*); AS bcl-2 doses are 0.5 μM (*a, e,* and *i*), 1 μM (b, f, j), 2 μM (*c. g.* and *k*), and 5 μM (*d, h,* and *l*). Scramble MBO dose is 1 μM in all three cell lines. Data are expressed as the percentage of growth inhibition in reference to the growth of untreated control cells. Each *bar,* the percentage of growth inhibition, as indicated in the respective legends. *Bars* on the *left,* the sum of the effects of the individual agents and the expected percentage of growth inhibition if oligonucleotides are additive when used in combination. Total height of the *solid bar* on the *right,* the actual observed growth inhibition when oligonucleotides were used in combination. Therefore, the differences between the heights of the *paired bars* reflect the magnitude of synergism of growth inhibition. The data represent means and SEs of triplicate determination of at least two experiments.

products RIα and bcl-2 by Western blotting analysis. RIα protein expression was inhibited in GEO cells by AS RIα, as shown previously (34), whereas it was unaffected by AS bcl-2 (data not shown). Fig. 2 illustrates the effect on bcl-2 expression of the different oligonucleotides at concentrations close to the IC_{50}. AS bcl-2 (5 μM) inhibited the total bcl-2 protein expression,
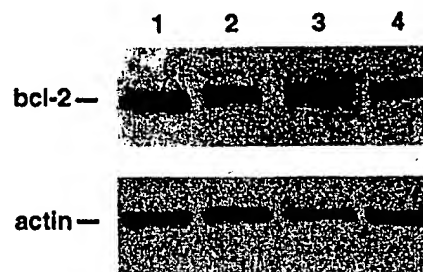

Fig. 2 Western blot analysis of bcl-2 and actin protein levels. Treatments were as follows: *Lane 1,* untreated; *Lane 2,* AS bcl-2, 5 μM; *Lane 3,* AS RIα, 1 μM; and *Lane 4,* AS bcl-2 μM plus AS RIα μM.

reducing >70% its nonphosphorylated form, whereas the AS RIα (1 μM) prevalently increased the relative amount of phosphorylated bcl-2 without affecting the total amount of protein. Interestingly, the combined treatment with the two oligonucleotides caused an additive effect, markedly decreasing the total amount of bcl-2 protein and inducing a prevalently phosphorylated bcl-2 (Fig. 2). Combination of either AS with an equal dose of the relative control sequence did not affect the results obtained with each AS alone (data not shown).

Cooperative Effect of the Two Oligonucleotides on Apoptosis. We measured the effect of different AS treatments on the induction of apoptosis *in vitro.* Previously, we have shown by flow cytometry that the apoptotic cells are ~4% in untreated GEO cells and ~6% in untreated OVCAR-3 cells (35). In GEO, we have observed that the AS RIα (1 μM) is able to double the rate of cells undergoing apoptosis as compared with untreated control cells, whereas the AS bcl-2 causes a 1.5-fold increase in the apoptotic index at 1 μM and a 4-fold increase at 2 μM, respectively (Fig. 3*A*). When the MBO AS RIα was combined with the PS-AS bcl-2, either 1 μM or 2 μM, they determined a supraadditive apoptotic effect, causing an 8-fold and an ~13-fold increase in apoptotic index, respectively. Conversely, the addition of scramble MBO to either dose of AS bcl-2 did not modify the effect observed with the latter oligonucleotides alone. A supraadditive effect, although less pronounced, was also observed in ovarian OVCAR-3 cells (Fig. 3*B*). In fact, AS RIα (1 μM) caused a 1.8-fold increase in the apoptotic index and AS bcl-2 (1 μM and 2 μM), caused a 1.5-fold and a 2.4-fold increase, respectively. The AS RIα combined with the AS bcl-2 (1 μM and 2 μM) induced a 4.3-fold and a 6.1-fold increase in apoptosis, respectively. The scramble MBO was not effective, causing a less-than-additive effect when added to either dose of AS bcl-2 (Fig. 3*B*).

Cooperative Antitumor Activity *in Vivo.* We evaluated whether the cooperative antitumor effect observed *in vitro* could be reproduced *in vivo* using the model of nude mice bearing GEO xenografts. Seven days after tumor injection, groups of 10 mice were randomly selected to receive different treatments, including p.o. AS RIα, i.p. AS bcl-2, i.p. mismatch bcl-2 oligonucleotide, or the combination of either p.o. AS RIα plus i.p. AS bcl-2 or p.o. AS RI plus i.p. mismatch bcl-2 oligonucleotide (described in "Materials and Methods"). Treatment was performed for 5 days a week, for 3 consecutive weeks, up to day 25.

A

B
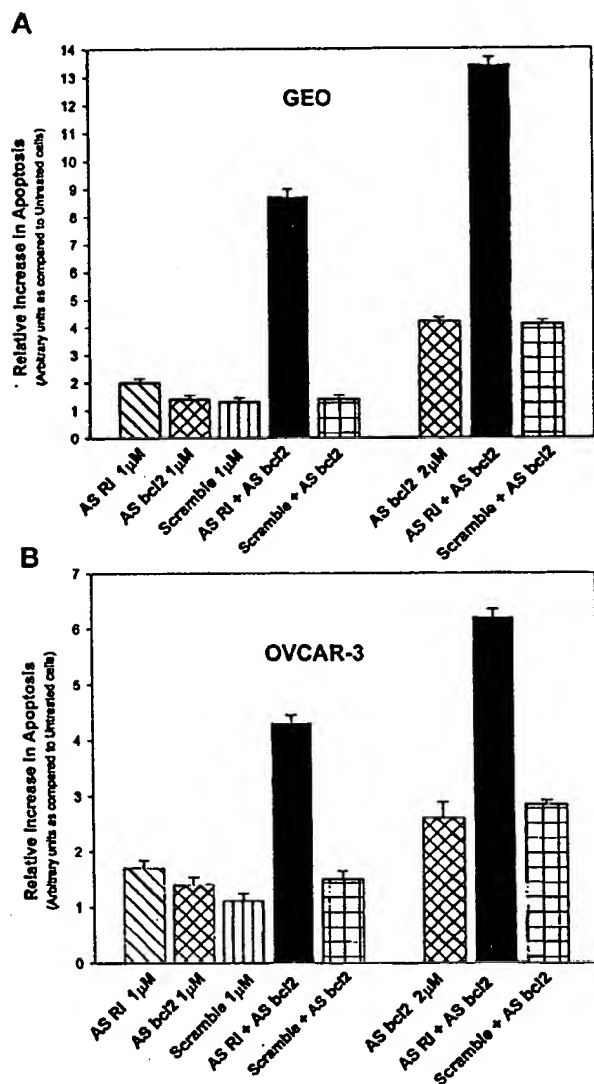
Fig. 3 Effect of the AS RIα MBO, scramble MBO, and AS bcl-2 on the induction of apoptosis in GEO (*A*) and OVCAR-3 (*B*) cells. Treatments were carried out as described in "Materials and Methods." Doses of each oligonucleotide alone and in combination are reported. Data are expressed as apoptotic index (*AI*), which represents the ratio between the absorbance in optical density of treated cells and that of untreated cells normalized for the same number of cells. Therefore, results for each treatment are intended as relative to control untreated cells, referred to as 1. The percentage of apoptotic cells is ~4% in untreated GEO cells and ~6% in untreated OVCAR-3 cells, as determined by flow cytometry (35). The data represent means and SEs of quadruplicate determination of at least two experiments.

As illustrated in Fig. 4*A*, treatment with either AS RIα or AS bcl-2 alone inhibited tumor growth as compared with untreated mice. In fact, by day 35, each AS oligonucleotide caused a 80–90% inhibition of tumor growth as compared with control untreated mice. However, shortly after the end of treatment, tumors resumed the growth rate observed in untreated mice, achieving their same size by day 56. We have shown previously

A

B
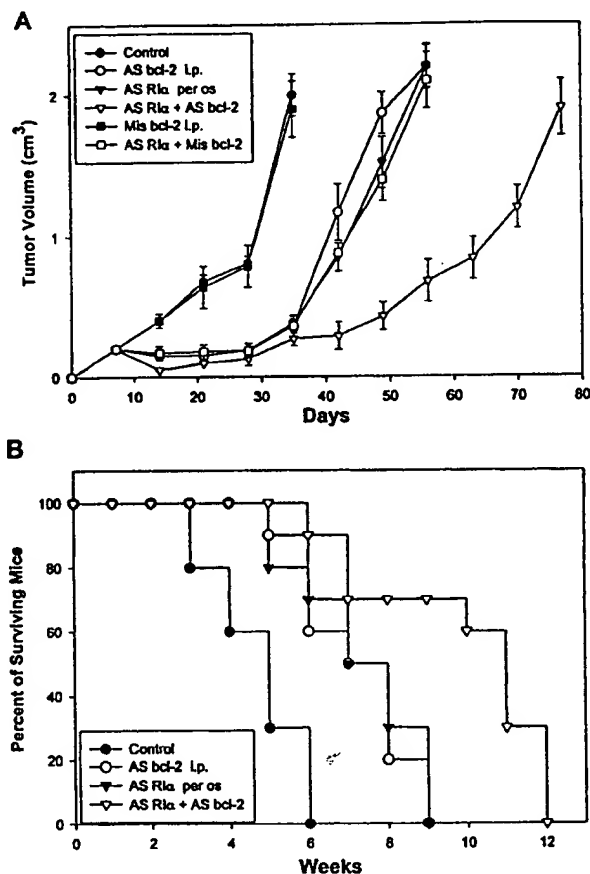
Fig. 4 Effect of different oligonucleotides on GEO tumor growth and on mice survival. *A*, effect of p.o. AS RIα MBO, i.p. AS bcl-2, i.p. mismatch bcl-2 (*mis bcl-2*) alone and in combination. Each group of 10 mice received three cycles of treatment as described in "Materials and Methods." *B*, effect on mice survival of the different treatments described in *A*. The Mantel-Cox log-rank test was used to evaluate the statistical significance of the results. The difference between the treatment with either AS RIα or AS bcl-2 is significant as compared with control untreated (*P* < 0.001). The difference between the treatment with AS RIα plus AS bcl-2 is significant as compared with control untreated or mismatch bcl-2-treated mice (*P* < 0.0001), with AS bcl-2-treated mice (*P* < 0.001), or with AS RIα-treated mice (*P* < 0.001).

that a scramble MBO oligonucleotide is ineffective in the same tumor model (10). Similarly, the mismatch control bcl-2 oligonucleotide was ineffective (Fig. 4*A*). When the p.o. AS RIα was used in combination with the AS bcl-2, we observed a marked and sustained inhibition of tumor growth. In fact, tumors achieved an average volume of about 2 cm³ 6 weeks later than mice that were untreated or treated with mismatch control and 3 weeks later than animals treated with each single AS alone. In contrast, treatment with AS RIα plus mismatch bcl-2 caused an antitumor effect similar to that obtained with the AS RIα alone. Within ~6 weeks, GEO tumors reached a size not compatible with normal life in all untreated mice and in mice treated with the control bcl-2 oligonucleotide (Fig. 4*B*). A significant increase in survival was observed in mice groups treated with

Table 2 Immunohistochemical analysis of GEO tumors subsequent to treatment with different oligonucleotides

Analysis was performed at the end of the second cycle of treatment (day 21) on three different tumor specimens randomly selected in each group. Numbers represent the percentage of positive cells staining for each antigen and for apoptotic nuclei or the number of microvessels/field (see "Materials and Methods"). Numbers in parenthesis represent the SD in the measurement of each biological parameter. The intensity of staining is represented by a score from + to +++.

	Ki67	RIα	Vessels	Apoptosis
Control	60% (±5) ++	60% (±4) +++	10 (±1) ++	3 (±1)
AS bcl-2	40% (±2) +	60% (±5) +++	7 (±1) +	7 (±1)
AS RIα	40% (±5) +	20% (±3) +	7 (±1) +	4 (±1)
AS bcl-2 + AS RIα	20% (±4) +	10% (±5) +	3 (±1) +	11 (±1)

either AS RIα or AS bcl-2 alone, as compared with control mice ($P < .001$). A remarkable result was obtained in mice treated with AS RIα plus AS bcl-2 in combination. In fact, the delayed GEO tumor growth observed in this group was accompanied by a prolonged life span in mice, significantly different when compared by log-rank test to controls ($P < .0001$), to either the AS alone-treated group ($P < .001$) or to the AS RIα plus mismatch bcl-2-treated group ($P < .001$). Approximately 60% of mice treated with AS RIα plus AS bcl-2 were still alive 11 weeks after tumor injection, representing the only animals alive at this time point. The combined treatment with AS RIα plus AS bcl-2 was well tolerated, inasmuch as no weight loss or other signs of acute or delayed toxicity were observed.

Effect on Angiogenesis, Proliferation, and Apoptosis *in Vivo*. Tumor specimens from the different groups of mice were examined by immunohistochemical analysis to evaluate the expression of a variety of biological parameters. Table 2 reports the results of the analyses performed on two tumor samples randomly selected in each group after the 2nd week of treatment on day 21. Treatment with AS RIα was able to inhibit the expression of both the target protein, RIα, and Ki67, a protein related to cell proliferation, confirming our previous results (10). Interestingly, AS bcl-2 inhibited Ki67 but did not affect RIα expression (Table 1). Additional inhibition of RIα and Ki67 expression was observed when the MBO AS RIα was used in combination with the AS bcl-2 (Table 2).

We quantified by immunohistochemistry the tumor-induced vascularization as microvessel count in the most intense areas of neovascularization using an anti-factor VIII related-antigen MAb (31). As reported in Table 2, the AS RIα as well the AS bcl-2 caused inhibition of specific vessel staining, as compared with samples from untreated mice. Combined treatment with AS RIα and AS bcl-2 inhibited by >70% the vessel formation in GEO tumors (Table 2).

Finally, we studied the effect of treatment on the induction of apoptosis *in vivo* by a modified TdT-mediated nick end labeling technique (32). We observed that the i.p. AS bcl-2 was very effective after only 2 weeks of treatment, doubling the percentage of cells undergoing apoptosis, whereas at the same time point, p.o. AS RIα only slightly increased apoptosis. Com-

bination of two oligonucleotides caused an additive effect on apoptosis.

DISCUSSION

Increased tumor expression of bcl-2 and other members of the antiapoptotic bcl-2 family as well as reduced expression of proapoptotic proteins are considered among the major determinants of resistance to chemotherapeutic drugs and radiotherapy and the persistence of microscopic proliferating clones, indicating a poor prognosis in several types of cancer (11, 12). Blockade of bcl-2 expression and function may represent a relevant therapeutic strategy, and AS bcl-2 oligonucleotides have been successful in several preclinical and clinical studies (15–20). Several studies suggest that PKA may play a major role in the control of bcl-2-dependent apoptosis. The PKAI isoform is involved in the transduction and amplification of a variety of mitogenic signals from cell membrane to core cellular machineries (2). Recently, different studies have linked PKA to the apoptotic machinery. For instance, microtubule-damaging agents determine apoptosis by a serine kinase-dependent phosphorylation of bcl-2. Both PKA and JNK have shown the ability to phosphorylate the specific serine-70 residue of bcl-2, to induce the cleavage of PARP, to activate caspase 3, and, finally, to cause apoptosis (21, 23, 24). It has also been demonstrated that selective inhibitors of PKAI and inducers of PKAIIβ, such as AS RIα, cAMP analogues, or selective unhydrolizable cAMP analogues, can inactivate bcl-2 and induce activation of the caspases cascade, cleavage of PARP, and apoptosis (24–26). A recent study has shown that a PKAII isoform is targeted by an anchoring protein to phosphorylate and inactivate BAD and that a selective inhibitor of PKAI activation may prevent this effect (36), suggesting that PKAI may be involved also in BAD functions. In this regard, an even more direct link between the apoptotic machinery and PKAI has been unraveled by Yang *et al.* (37), showing that PKAI subunit RIα is directly bound to cytochrome *c* oxidase subunit Vb. Pharmacological inhibition of PKAI causes cytochrome *c* release and apoptosis, whereas overexpression of mutant PKAI, harboring a defective RIα, prevents these events (37).

This large body of experimental evidence would imply that the selective blockade of bcl-2, combined with a selective inhibition of the multifunctional signaling protein PKAI, could trigger the apoptosis and suppress the transduction of survival signals, thus leading to cancer cell growth inhibition.

To experimentally demonstrate this hypothesis, in the present study we have used two AS oligonucleotides that have shown their therapeutic potential in different preclinical and clinical settings. The PS-AS bcl-2 has shown antitumor activity in human melanoma and prostate cancer models in nude mice (15, 16). The same compound has shown antitumor and chemosensitizing activity in Phase I/II clinical trials in melanoma, non-Hodgkin's lymphomas, and prostate cancer subsequent to i.v. as well as s.c. administration (18–20). The AS RIα is a novel 2'-O-methyl-modified MBO that has shown antitumor activity *in vitro* and *in vivo* in a variety of tumor models in nude and severe combined immunodeficient mice (7, 8). We and others have also shown that this compound has good bioavailability and antitumor activity in the same tumor models after

p.o. administration (9, 10). Recently, the AS RIα MBO has completed a Phase I trial in cancer patients with negligible toxicity (8).

We have here demonstrated that both the AS bcl-2 and the MBO AS RIα are able to inhibit, in a dose-dependent fashion, the anchorage-independent growth of GEO colon, ZR-75-1 breast and OVCAR-3 ovarian cancer cells. When used in combination at doses causing only moderate inhibition as single agents, they show a marked cooperative growth-inhibitory effect, allowing the suppression of colony formation in agar. The fact that the cooperative antiproliferative effect can be obtained regardless of the schedule of administration used, further supports the hypothesis that the two pathways are distinct yet related to each other. Inhibition of bcl-2 expression as well as bcl-2 phosphorylation are critical events in the commitment to apoptosis. In this study we have demonstrated that AS RIα causes bcl-2 phosphorylation in GEO cells and that combination with AS bcl-2 markedly reduces the total expression of bcl-2, which then results mostly in a phosphorylated form. These data are in agreement with previous observations, showing that AS RIα affects bcl-2 expression and causes bcl-2 phosphorylation (23, 34). More importantly, the combination of AS oligonucleotides caused a remarkable cooperative effect on the induction of apoptosis *in vitro*. These data suggest that combined blockade of PKAI and bcl-2 imbalance antiapoptotic cellular mechanisms, causing cell death. We have then evaluated whether the effects *in vitro* could be obtained also *in vivo* in nude mice bearing GEO tumors. Previously, we have shown the inhibitory effect of p.o. MBO AS RIα in GEO tumor xenografts. In this study we have demonstrated the cooperative antitumor effect of p.o. AS RIα in combination with i.p. PS-AS bcl-2 after 3 weeks of treatment. The growth-inhibitory effect was sustained, lasting for several weeks after treatment withdrawal and causing a remarkable increase in the survival of treated mice. In fact, ~60% of mice treated with the two oligonucleotides in combination were still alive 11 weeks after tumor injection. We and others have shown previously that either the p.o. AS RIα or an AS bcl-2 cooperate with paclitaxel in inhibiting the growth of tumors in mice (10, 17). Interestingly, in the present study we have observed that the addition of paclitaxel to both ASs together minimally increases the antiproliferative and apoptotic effect obtained by the two oligonucleotides in combination.[4] We have demonstrated that the p.o. AS RIα selectively inhibits the expression of its target in the tumor. In fact, although bcl-2 also inhibits tumor growth and reduces expression of the proliferation-related protein Ki67, it does not affect RIα expression. The critical role of tumor-induced neovascularization in neoplastic development, progression, and metastasis has been emphasized in recent years (38). In the present study, we have shown that either oligonucleotide alone was active in inhibiting vessel staining after only 2 weeks of treatment and that combined treatment with the two oligonucleotides reduced >70% vessel formation in GEO tumors. Finally, we have observed that AS bcl-2 induced a more-than-double increase in apoptosis, whereas the AS RIα was modestly effective after 2 weeks of

treatment. Interestingly, the two oligonucleotides in combination caused an additive apoptotic effect.

It has been proposed that novel therapeutic strategies should target the expression and function of mitogenic and antiapoptotic proteins as well as angiogenesis, causing apoptosis and/or inducing a status of tumor dormancy (39). In the same fashion it can be proposed that multiple selective inhibitors may be used to interfere with different growth-escape pathways. In this study, we have demonstrated that two AS oligonucleotides, a PS-oligonucleotide targeting bcl-2 and a hybrid MBO targeting PKAI, cooperatively inhibit the growth of and induce apoptosis in human cancer cells *in vitro*. The same compounds administered i.p. and p.o. for a short term caused a marked antitumor effect, accompanied by the inhibition of angiogenesis and the induction of apoptosis in nude mice bearing established GEO tumors.

This study represents the first demonstration that two oligonucleotides targeting two preeminent signaling pathways have a cooperative antitumor activity *in vivo* and further supports the hypothesis of a direct link between PKA and bcl-2. Because both AS RIα and AS bcl-2 have shown promising results in clinical trials and also can be used by p.o. and s.c. routes, respectively, our study provides the rationale to translate a feasible and novel treatment in cancer patients.

ACKNOWLEDGMENTS

REFERENCES

1. Cho-Chung, Y. S., Pepe, S., Clair, T., Budillon, A., and Nesterova, M. cAMP-dependent protein kinase: role in normal and malignant growth. Crit. Rev. Oncol. Hematol., *21:* 33–61, 1995.

2. Ciardiello, F., and Tortora, G. Interactions between the epidermal growth factor receptor and type I protein kinase A: biological significance and therapeutic implications. Clin. Cancer Res., *4:* 821–828, 1998.

3. Tortora, G., Damiano, V., Bianco, C., Baldassarre, G., Bianco, A. R., Lanfrancone, L., Pelicci, P. G., and Ciardiello, F. The RIα subunit of protein kinase A (PKA) binds to Grb2 and allows PKA interaction with the activated EGF-receptor. Oncogene. *14:* 923–928, 1997.

4. Cho-Chung, Y. S. Antisense oligonucleotide inhibition of serine/threonine kinases: an innovative approach to cancer treatment. Pharmacol. Ther., *82:* 437–449, 1999.

5. Nesterova, M., and Cho-Chung, Y. S. A single-injection protein kinase A-directed antisense treatment to inhibit tumor growth. Nat. Med., *1:* 528–533, 1997.

6. Agrawal, S., and Zhao, Q. Antisense therapeutics. Curr. Opin. Chem. Biol., *2:* 519–528, 1998.

7. Tortora, G., Caputo, R., Damiano, V., Bianco, R., Pepe, S., Bianco, A. R., Jiang, Z., Agrawal, S., and Ciardiello, F. Synergistic inhibition of human cancer cell growth by cytotoxic drugs and mixed backbone antisense oligonucleotide targeting protein kinase A. Proc. Natl. Acad. Sci. USA, *94:* 12586–12589, 1997.

8. Chen, H. X., Marshall, J. L., Ness, E., Martin, R., Dvorchik, B., Rizvi, N., Marquis, J., McKinlay, M., Dahut, W., and Hawkins, M. J. A safety and pharmacokinetic study of a mixed-backbone oligonucleotide (GEM 231) targeting the type I protein kinase A by 2-hour infusions in patients with refractory solid tumors. Clin. Cancer Res., *6:* 1259–1266, 2000.

9. Wang, H., Cai, Q., Zeng, X. Yu, D., Agrawal, S., and Zhang, R. Antitumor activity and pharmacokinetics of a mixed-backbone oligonu-

---

[4] Unpublished results.

cleotide targeted to the RIα subunit of protein kinase A following oral administration. Proc. Natl. Acad. Sci. USA, 96: 13989–13994, 1999.

10. Tortora, G., Bianco, R., Damiano, V., Fontanini, G., De Placido, S., Bianco, A. R., and Ciardiello, F. Oral antisense that targets protein kinase A cooperates with Taxol and inhibits tumor growth, angiogenesis, and growth factor production. Clin. Cancer Res., 6: 2506–2512, 2000.

11. Reed, J. Dysregulation of apoptosis in cancer. J. Clin. Oncol., 17: 2941–2953, 1999.

12. Sellers, W. R., and Fisher, D. E. Apoptosis and cancer drug targeting. J. Clin. Investig., 104: 1655–1661, 1999.

13. Gross, A., McDonnell, J. M., and Korsmeyer, S. J. BCL-2 family members and the mitochondria in apoptosis. Genes Dev., 13: 1899–1911, 1999.

14. Blagosklonny, M. V., and Fojo, T. Molecular effects of paclitaxel: myths and reality (a critical review). Int. J. Cancer, 83: 151–156, 1999.

15. Jansen, B., Schlagbauer-Wadl, H., Brown, B. D., Bryan, R. N., van Elsas, A., Muller, M., Wolff, K., Eichler, H. G., and Pehamberger, H. Bcl-2 antisense therapy chemosensitizes human melanoma in SCID mice. Nat. Med., 4: 232–234, 1998.

16. Gleave, M., Tolcher, A., Miyake, H., Nelson, C., Brown, B., Beraldi, E., and Goldie, J. Progression to androgen independence is delayed by adjuvant treatment with antisense bcl-2 oligodeoxynucleotides after castration in the LNCaP prostate tumor model. Clin. Cancer Res., 5: 2891–2898, 1999.

17. Miyake, H., Tolcher, A., and Gleave, M. Chemosensitization and delayed androgen independent recurrence prostate cancer with the use of antisense bcl-2 oligodeoxynucleotides. J. Natl. Cancer Inst. (Bethesda), 92: 34–41, 2000.

18. Jansen, B., Wacheck, V., Heere-Ress, E., Schlagbauer-Wadl, H., Hoeller, C., Lucas, T., Hoermann, M., Hollenstein, U., Wolff, K., and Pehamberger, H. A. Chemosensitization of malignant melanoma by BCL2 antisense therapy. Lancet, 356: 1728–1733, 2000.

19. Waters, J. S., Webb, A., Cunningham, D., Clarke, P., Raynaud, F., di Stefano, F., and Cotter, F. E. Phase I clinical and pharmacokinetic study of bcl-2 antisense oligonucleotide therapy in patients with non-Hodgkin's lymphoma. J. Clin. Oncol., 18: 1812–1823, 2000.

20. Morris, M. J., Tong, W., Osman, I., Maslak, P., Kelly, W. K., Terry, K., Rosen, N., and Scher, H. I. A Phase I/IIA dose-escalating trial of bcl-2 antisense (G3139) treatment by 14-day continuous intravenous infusion (CI) for patients with androgen-independent prostate cancer or other advanced solid tumor malignancies. Proc. Am. Soc. Clin. Oncol., 18: 323, 1999.

21. Yamamoto, K., Ichijo, H., and Korsmeyer, S. J. BCL-2 is phosphorylated and inactivated by an ASK1/Jun N-terminal protein kinase pathway normally activated at G(2)/M. Mol. Cell Biol., 19: 8469–8478, 1999.

22. Downward, J. Mechanisms and consequences of activation of protein kinase B/Akt. Curr. Opin. Cell Biol., 10: 262–267, 1998.

23. Srivastava, R. K., Srivastava, A. R., Korsmeyer, S. J., Nesterova, M., Cho-Chung, Y. S., and Longo, D. L. Involvement of microtubules in the regulation of Bcl2 phosphorylation and apoptosis through cAMP-dependent protein kinase. Mol. Cell Biol., 18: 3509–3517, 1998.

24. Srivastava, R. K., Mi, Q. S., Hardwick, J. M., and Longo, D. L. Deletion of the loop region of Bcl-2 completely blocks paclitaxel-induced apoptosis. Proc. Natl. Acad. Sci. USA, 96: 3775–3780, 1999.

25. Srivastava, R. K., Srivastava, A. R., Seth, P., Agrawal, S., and Cho-Chung, Y. S. Growth arrest and induction of apoptosis in breast cancer cells by antisense depletion of protein kinase A-RI α subunit:

p53-independent mechanism of action. Mol. Cell Biochem., 195: 25–36, 1999.

26. Itano, Y., Ito, A., Uehara, T., and Nomura, Y. Regulation of bcl-2 protein expression in human neuroblastoma SH-Sy5Y cells: positive and negative effects of protein kinases C and A respectively. J. Neurochem., 67: 131–137, 1996.

27. Padmapriya, A. A., Tang, J. Y., and Agrawal, S. Large-scale synthesis, purification, and analysis of oligodeoxynucleotide phosphorothioates. Antisense Res. Dev., 4: 185–199, 1994.

28. Ciardiello, F., Pepe, S., Bianco, C., Baldassarre, G., Ruggiero, A., Bianco, C., Selvam, M. P., Bianco, A. R., and Tortora, G. Downregulation of RIα subunit of the cAMP-dependent protein kinase induces growth inhibition of human mammary epithelial cells transformed by c-Ha-ras and c-erbB2 protooncogenes. Int. J. Cancer, 53: 438–443, 1993.

29. Ciardiello, F., Damiano, V., Bianco, R., Bianco, C., Fontanini, G., De Laurentiis, M., De Placido, S., Mendelsohn, J., Bianco, A. R., and Tortora, G. Antitumor activity of combined blockade of epidermal growth factor receptor and protein kinase A. J. Natl. Cancer Inst. (Bethesda), 88: 1770–1776, 1996.

30. Fontanini, G., De Laurentiis, M., Vignati, S., Chinè, S., Lucchi, M., Silvestri, V., Mussi, A., De Placido, S., Tortora, G., Bianco, A. R., Gullick, W., Angeletti, C. A., Bevilacqua, G., and Ciardiello, F. Evaluation of epidermal growth factor-related growth factors and receptors and of neoangiogenesis in completely resected stage I-IIIA non-small cell lung cancer: amphiregulin and microvessel count are independent prognostic indicators of survival. Clin. Cancer Res., 4: 241–249, 1998.

31. Weidner, N. Current pathologic methods for measuring intratumoral microvessel density within breast carcinoma and other solid tumors. Breast Cancer Res. Treat., 36: 169–180, 1995.

32. Cuello-Carrion, F. D., and Ciocca, D. R. Improved detection of apoptotic cell using a modified in situ TUNEL technique. J. Histochem. Cytochem., 47: 837–839, 1999.

33. Mantel, N. Evaluation of survival data and two new rank order statistics arising in its consideration. Cancer Chemother. Rep., 50: 163–170, 1996.

34. Tortora, G., Caputo, R., Pomatico, G., Pepe, S., Bianco, A. R., Agrawal, S., Mendelsohn, J., and Ciardiello, F. Cooperative inhibitory effect of novel mixed backbone oligonucleotide targeting protein kinase A in combination with docetaxel and anti-EGFR-receptor antibody on human breast cancer cell growth. Clin. Cancer Res., 5: 875–881, 1999.

35. Ciardiello, F., Bianco, R., Damiano, V., De Lorenzo, S., Pepe, S., De Placido, S., Fan, Z., Mendelsohn, J., Bianco, A. R., and Tortora, G. Antitumor activity of sequential treatment with topotecan and anti-epidermal growth factor receptor monoclonal antibody C225. Clin. Cancer Res., 5: 909–916, 1999.

36. Harada, H., Becknell, B., Wilm, M., Mann, M., Huang, L. J., Taylor, S. S., Scott, J. D., and Korsmeyer, S. J. Phosphorylation and inactivation of BAD by mitochondria-anchored protein kinase A. Mol. Cell, 3: 413–422, 1999.

37. Yang, W. L., Iacono, L., Tang, W. M., and Chin, K. V. Novel function of the regulatory subunit of protein kinase A: regulation of cytochrome c oxidase activity and cytochrome c release. Biochemistry, 37: 14175–14180, 1998.

38. Hahnfeldt, P., Panigrahy, D., Folkman, J., and Hlatky, L. Tumor development under angiogenic signaling: a dynamical theory of tumor growth, treatment response, and postvascular dormancy. Cancer Res., 59: 4770–4775, 1999.

39. Holmgren, L., O'Reilly, M. S., and Folkman, J. Dormancy of micrometastases: balanced proliferation and apoptosis in the presence of angiogenesis suppression. Nat. Med., 1: 149–153, 1995.

Advances in Brief

Antisense TRPM-2 Oligodeoxynucleotides Chemosensitize Human Androgen-independent PC-3 Prostate Cancer Cells Both *in Vitro* and *in Vivo*[1]

Hideaki Miyake, Kim N. Chi, and
Martin E. Gleave[2]

The Prostate Centre, Vancouver General Hospital, Vancouver, British
Columbia, V6H 3Z6 Canada [H. M., K. N. C., M. E. G.], and
Division of Urology, University of British Columbia, Vancouver,
British Columbia, V5Z 3J5 Canada [M. E. G.]

Abstract

Although numerous chemotherapeutic regimens have been evaluated for patients with hormone-refractory prostate cancer, none has improved survival. Testosterone-repressed prostate message-2 (TRPM-2), which is highly up-regulated after androgen withdrawal and during androgen-independent progression in prostate cancer, has been shown to inhibit apoptosis induced by various kinds of stimuli. The objectives in this study were to test whether antisense (AS) oligodeoxynucleotides (ODNs) targeted against *TRPM-2* enhance chemosensitivity in human androgen-independent prostate cancer PC-3 cells both *in vitro* and *in vivo*. Initially, the potency of 10 AS ODNs targeting various regions of the TRPM-2 mRNA were evaluated, and the AS ODN targeted to the *TRPM-2* translation initiation site (AS ODN#2) was found to be the most potent sequence for inhibiting TRPM-2 expression in PC-3 cells. Despite significant dose-dependent and sequence-specific suppression of TRPM-2 expression, AS ODN#2 had no effect on growth of PC-3 cells both *in vitro* and *in vivo*. However, pretreatment of PC-3 cells with AS ODN#2 significantly enhanced chemosensitivity of Taxol (paclitaxel) and mitoxantrone *in vitro*. Characteristic apoptotic DNA laddering and cleavage of poly(ADP-ribose) polymerase were observed after combined treatment with AS ODN#2 plus paclitaxel or mitoxantrone but not with either agent alone. *In vivo* administration of AS ODN#2 plus either paclitaxel or mitoxantrone significantly decreased PC-3 tumor volume by 80 or 60%, respectively, compared with mismatch control ODN plus either paclitaxel or mitoxantrone. In addition, terminal deoxynucleotidyl transferase-mediated nick end labeling staining revealed increased apoptotic cells in tumors treated

with AS ODN#2 plus paclitaxel or mitoxantrone. These findings confirm that TRPM-2 overexpression confers resistance to cytotoxic chemotherapy in prostate cancer cells and illustrates the potential utility of combined treatment with AS TRPM-2 ODN plus chemotherapeutic agents for patients with hormone-refractory prostate cancer.

Introduction

Prostate cancer is now the most commonly diagnosed malignancy and the second leading cause of cancer mortality in men in Western industrialized countries. Androgen withdrawal remains the only effective therapy for patients with advanced disease. Approximately 80% of patients achieve symptomatic and/or objective response after androgen ablation; however, progression to androgen independence ultimately occurs in almost all cases (1). Although numerous nonhormonal agents have been evaluated in patients with hormone-refractory prostate cancer, these agents have limited antitumor activity with an objective response rate of <20% and no demonstrated survival benefit (2). Therefore, novel therapeutic strategies targeting molecular mechanisms mediating resistance to conventional agents must be developed to make a significant impact on survival.

Advances in the field of nucleic acid chemistry offers one attractive strategy to design AS[3] ODN-based therapeutic agents that specifically hybridize with complementary mRNA regions of a target gene and thereby inhibit gene expression by forming RNA/DNA duplexes (3). Rapid intracellular degradation of ODNs is a potential disadvantage of AS ODN therapy, but this problem can be overcome by substituting a nonbridging phosphoryl oxygen of DNA with a sulfur to create a phosphorothioate backbone, which stabilizes the ODN to nuclease digestion (4). Recently, several antisense ODNs targeted against specific genes involved in neoplastic progression have been evaluated as potential therapeutic agents (5–8). Collectively, these findings identify AS ODNs as a novel class of antineoplastic agents when designed for appropriate molecular targets. However, because numerous genes are involved in tumor progression, inhibition of a single target gene will likely be insufficient to inhibit tumor progression in a meaningful way. In fact, combined use of AS ODN with other compounds, such as chemotherapeutic

agents, have been demonstrated to produce more potent antineoplastic effects in some tumor model systems (9–12).

TRPM-2, also known as clusterin or sulfated glycoprotein-2, was first isolated from ram rete testes fluid (13) and plays important roles in various pathophysiological processes, including tissue remodeling, reproduction, lipid transport, complement regulation, and apoptosis (14). Because TRPM-2 expression is increased in various benign and malignant tissues undergoing apoptosis, it has been regarded as a marker for cell death (15–18). Recent studies, however, provide conflicting findings regarding the relationship between TRPM-2 up-regulation and increased apoptotic activity (19–21). Similarly, TRPM-2 expression increases in regressing normal prostatic epithelial cells (22, 23) and prostate cancer xenografts (24, 25) after treatment with various apoptotic stimuli and is associated with cell survival and disease progression in prostate cancer (26–30). We have reported recently that an increase in TRPM-2 expression after androgen ablation accelerates tumor progression by inhibiting castration (31)- and chemotherapy[4]-induced apoptosis. However, the functional significance of TRPM-2 expression in established AI human prostate cancer has not been examined.

In the present study, we designed and screened 10 phosphorothioate AS ODNs targeted against the human *TRPM-2* gene to identify potent ODN sequences that specifically inhibit TRPM-2 expression in human AI prostate cancer PC-3 cells. We then tested whether AS TRPM-2 ODN can enhance the response of PC-3 cells to either Taxol (paclitaxel) or mitoxantrone both *in vitro* and *in vivo*.

Materials and Methods

Tumor Cell Line. PC-3, derived from human prostate cancer, was purchased from the American Type Culture Collection (Rockville, MD). Cells were maintained in DMEM (Life Technologies, Inc., Gaithersburg, MD) supplemented with 5% heat-inactivated FCS.

Chemotherapeutic Agents. Paclitaxel and mitoxantrone were purchased from Sigma Chemical Co. (St. Louis, MO) and Wyeth-Ayerst, Inc. (Montreal, Canada), respectively. Stock solutions of paclitaxel and mitoxantrone (1 mg/ml) were prepared with DMSO and diluted with PBS to the required concentrations before each *in vitro* experiment. Polymeric micellar paclitaxel used in these *in vivo* studies was generously supplied by Dr. Helen M. Burt (Faculty of Pharmaceutical Sciences, University of British Columbia, Vancouver, Canada).

AS TRPM-2 ODN. Phosphorothioate ODNs used in this study were obtained from the Nucleic Acid-Protein Service Unit, University of British Columbia (Vancouver, Canada). The sequences of 10 AS TRPM-2 ODNs (AS ODN#1 to AS ODN#10) and a two-base TRPM-2 MM ODN used as a control (MM Co. ODN) were as follows: AS ODN#1, 5'-TG-GAGTCTTTGCACGCCTCGG-3'; AS ODN#2, 5'-CAGCAG-CAGAGTCTTCATCAT-3'; AS ODN #3, 5'-ATTGTCTGAG-

ACCGTCTGGTC-3'; AS ODN#4, 5'-CCTTCAGCTTTGT-CTCTGATT-3'; AS ODN#5, 5'-AGCAGGGAGTCGATG-CGGTCA-3'; AS ODN#6, 5'-ATCAAGCTGC-GGACGATG-CGG-3'; AS ODN#7, 5'-GCAGGCAGCCCGTGGAGTTGT-3'; AS ODN#8, 5'-TTCAGCTGCTCCAGCAAGGAG-3'; AS ODN#9, 5'-AATTTAGGGTT- CTTCCTGGAG-3'; AS ODN#10, 5'-GCTGGGCGGAGTTGGGGGCCT-3'; and MM Co. ODN, 5'-CAGCAGCAGAGTATTTATCAT-3'.

Treatment of Cells with ODN. Lipofectin, a cationic lipid (Life Technologies, Inc.), was used to increase the ODN uptake of cells. PC-3 cells were treated with various concentrations of ODN after a preincubation for 20 min with 3 μg/ml lipofectin in serum-free OPTI-MEM (Life Technologies, Inc.). Four h after the beginning of the incubation, the medium containing ODN and lipofectin was replaced with standard culture medium described above.

Northern Blot Analysis. Total RNA was isolated from cultured PC-3 cells and PC-3 tumor tissues using the acid-guanidinium thiocyanate-phenol-chloroform method. Electrophoresis, hybridization, and washing conditions were carried out as reported previously (8). Human TRPM-2 and GAPDH cDNA probes were generated by reverse transcription-PCR from total RNA of human kidney using primers 5'-AAGGAAAT-TCAAAATGCTGTCAA-3' (sense) and 5'-ACAGACAA-GATCTCCCGGCACTT-3' (AS) for TRPM-2, and 5'-TGC-TTTTAAC-TCTGGTAAAGT-3' (sense) and 5'-ATATTTGG-CAGGTTTTTCTAGA-3' (AS) for GAPDH. The density of bands for TRPM-2 was normalized against that of GAPDH by densitometric analysis.
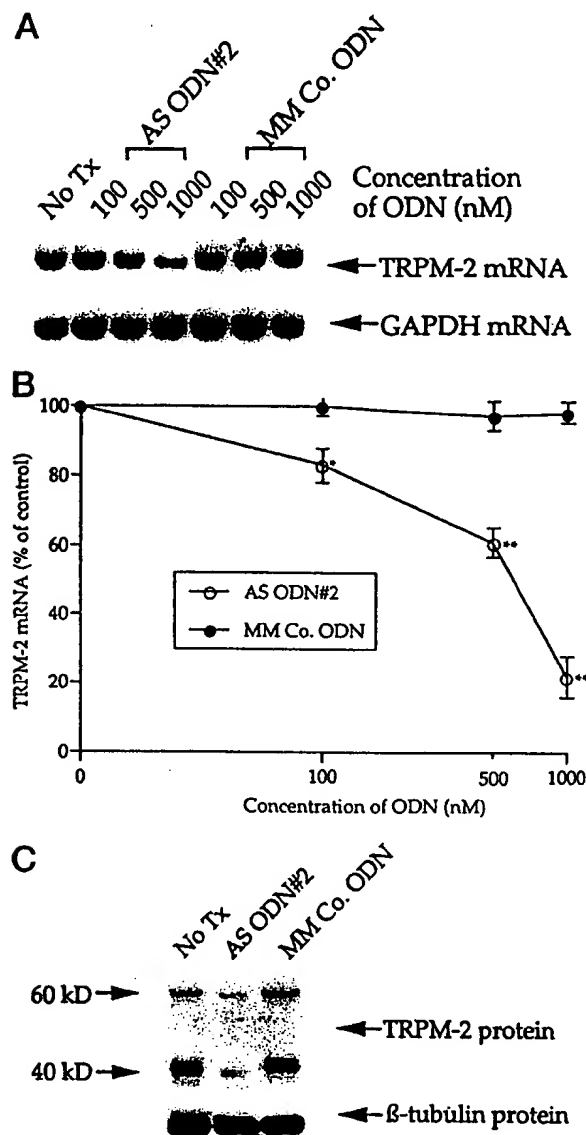
Western Blot Analysis. Samples containing equal amounts of protein (15 μg) from lysates of the cultured PC-3 cells and PC-3 tumors were electrophoresed on a SDS-polyacrylamide gel and transferred to a nitrocellulose filter. The filters were blocked in PBS containing 5% nonfat milk powder at 4°C overnight and then incubated for 1 h with a 1:400-diluted antihuman TRPM-2 goat polyclonal antibody (Santa Cruz Biotechnology, Inc.), 1:10000-diluted antirat β-tubulin mouse monoclonal antibody (Chemicon International Inc., Tumecula, CA), or 1:600-diluted antihuman PARP mouse monoclonal antibody (PharMingen, Mississauga, Ontario, Canada). The filters were then incubated for 30 min with horseradish peroxidase-conjugated antigoat or mouse IgG antibody (Amersham Life Science, Arlington Heights, IL), and specific proteins were detected using an enhanced chemiluminescence Western blotting analysis system (Amersham Life Science).

MTT Assay. The *in vitro* growth-inhibitory effects of AS TRPM-2 ODN plus paclitaxel or mitoxantrone on PC-3 cells were assessed using the MTT assay as described previously (32). Briefly, 1 × 10⁴ cells were seeded in each well of 96-well microtiter plates and allowed to attach overnight. Cells were then treated once daily with 500 nM ODN for 2 days. After ODN treatment, cells were treated with various concentrations of paclitaxel or mitoxantrone. After 48 h of incubation, 20 μl of 5 mg/ml MTT (Sigma Chemical Co.) in PBS were added to each well, followed by incubation for 4 h at 37°C. The formazan crystals were then dissolved in DMSO. The absorbance was determined with a microculture plate reader (Becton Dickinson Labware, Lincoln Park, NJ) at 540 nm. Absorbance values were normalized to the values obtained for the vehicle-treated cells to

---

[4] H. Miyake, C. Nelson, P. S. Rennie, and M. E. Gleave. Acquisition of chemoresistant phenotype by overexpression of the antiapoptotic gene, *TRPM-2*, in prostate cancer xenograft models. Cancer Res., in press, 2000.

A

B
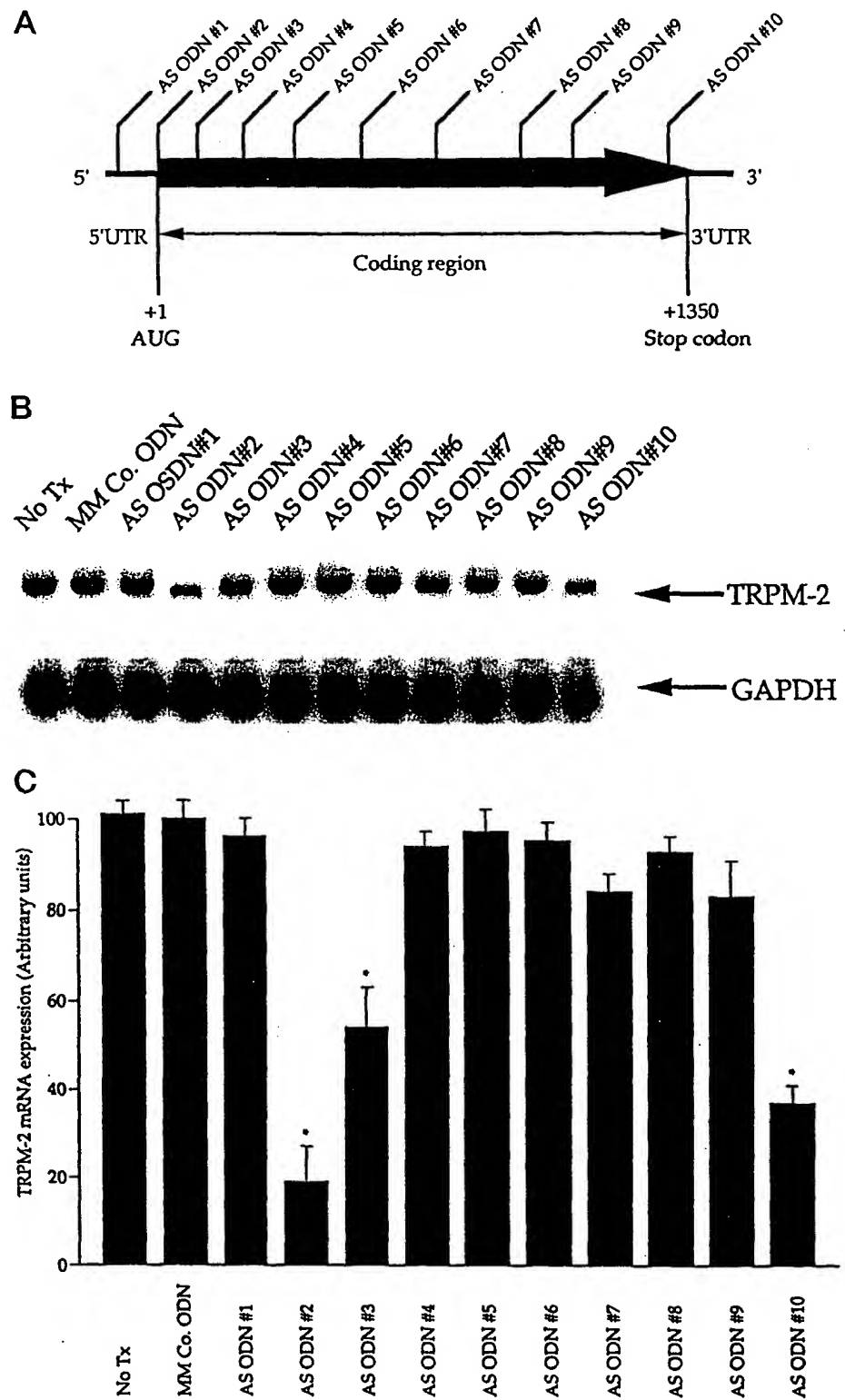
Fig. 1 Effects of various AS human TRPM-2 ODNs on TRPM-2 mRNA expression in PC-3 cells. *A,* schematic representation of the relative position of 10 phosphorothioate AS ODNs designed to hybridize with various regions of the *TRPM-2* gene. *UTR,* untranslated region. *B,* PC-3 cells were treated daily with AS TRPM-2 ODN or a two-base TRPM-2 MM ODN for 2 days. Total RNA was extracted from cultured cells and analyzed for TRPM-2 and GAPDH levels by Northern blotting. *No Tx,* untreated cells. *C,* quantitative analysis of TRPM-2 mRNA levels after normalization to GAPDH mRNA levels in PC-3 cells with AS TRPM-2 or MM Co. ODN was performed using a laser densitometer. *Columns.* means; *bars,* SD. ∗, differs from control ($P < 0.01$) by Student's *t* test.

C

A

Concentration
of ODN (nM)

◀—TRPM-2 mRNA

◀—GAPDH mRNA

B

C

60 kD ➤

40 kD ➤

◀—TRPM-2 protein

◀—ß-tubulin protein

Fig. 2 Sequence-specific and dose-dependent inhibition of TRPM-2 expression by AS TRPM-2 ODN in PC-3 cells. *A*, PC-3 cells were treated daily with various concentrations of AS TRPM-2 ODN (AS ODN#2: CAGCAGCAGA-GTCTTCATCAT) or a two-base TRPM-2 MM ODN (MM Co. ODN: CAGCA-GCAGAGTATTTAT-CAT) as a control for 2 days; total RNA was extracted from culture cells, and TRPM-2 and GAPDH levels were analyzed by Northern blotting. *No Tx*, untreated cells. *B*, quantitative analysis of TRPM-2 mRNA levels after normalization to GAPDH mRNA levels in PC-3 cells after treatment with various concentrations of AS ODN#2 or MM Co. ODN was performed by using laser densitometry. *Points*, means of triplicate analyses; *bars*, SD. ✶✶ and ✶, differ from control ($P < 0.01$ and $P < 0.05$, respectively) by Student's *t* test. *C*, PC-3 cells were treated daily with 1 μM AS ODN#2 or MM Co. ODN for 4 days, protein was extracted from culture cells, and TRPM-2 and β-tubulin protein levels were analyzed by Western blotting. *No Tx*, untreated cells.
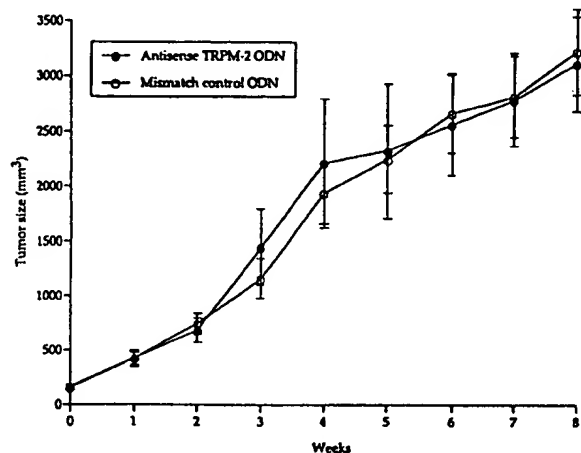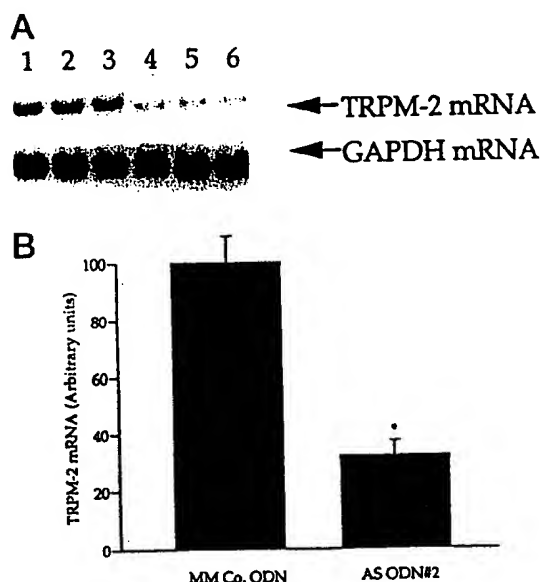

Fig. 3 Effects of AS TRPM-2 ODN administration on PC-3 tumor growth. When PC-3 tumors became ~1 cm in diameter, 10 mg/kg AS ODN#2 or MM Co. ODN were injected i.p. once daily for 28 days into each mouse. Tumor volume was measured once weekly and calculated by the formula: length × width × depth × 0.5236. *Points*, mean tumor volume in each experimental group containing eight mice; *bars*, SD.

determine the percentage of survival. Each assay was performed in triplicate.

DNA Fragmentation Analysis. Nucleosomal DNA degradation was analyzed as described previously with a minor modification (32). Briefly, 1×10^5 PC-3 cells were seeded in 5-cm culture dishes and allowed to adhere overnight. After treatment with ODN plus paclitaxel or mitoxantrone using the same schedule described above, cells were harvested and then lysed in a solution containing 100 mM NaCl, 10 mM Tris (pH 7.4), 25 mM EDTA, and 0.5% SDS. After the centrifugation, the supernatants were incubated with 300 μg/ml proteinase K for 5 h at 65°C and extracted with phenol-chloroform. The aqueous layer was treated with 0.1 volume of 3 M sodium acetate, and the DNA was precipitated with 2.5 volumes of 95% ethanol. After treatment with 100 μg/ml RNase A for 1 h at 37°C, the sample was electrophoresed on a 2% agarose gel and stained with ethidium bromide.

Assessment of *In Vivo* Tumor Growth. Approximately 1×10^6 PC-3 cells were inoculated s.c. with 0.1 ml of Matrigel (Becton Dickinson Labware, Bedford, MA) in the flank region of male athymic nude mice (BALB/c strain; Charles River Laboratory, Montreal, Quebec, Canada), 6–8 weeks of age, under methoxyfluorane anesthesia. When PC-3 tumors grew to 1 cm in diameter, usually 2–3 weeks after injection, mice were randomly selected for treatment with AS TRPM-2 ODN alone, MM Co. ODN alone, AS TRPM-2 ODN plus paclitaxel, MM Co. ODN plus paclitaxel, AS TRPM-2 ODN plus mitoxantrone, or MM Co. ODN plus mitoxantrone. Each experimental group consisted of eight mice. After randomization, 10 mg/kg AS TRPM-2 or MM Co. ODN was injected i.p. once daily into each mouse for 28 days. From days 10 to 14 and from days 24 to 28, 0.5 mg polymeric micellar paclitaxel or 0.03 mg mitoxantrone was administered once daily by i.v. injection. Tumor volume was measured once weekly and calculated by the formula:

A

1 2 3 4 5 6

◄—TRPM-2 mRNA

◄—GAPDH mRNA

B

Fig. 4 Effects of AS TRPM-2 ODN administration on TRPM-2 mRNA levels in PC-3 tumors in vivo. A, each of 3 PC-3 tumor-bearing mice were daily treated with AS ODN#2 or MM Co. ODN at a dose of 10 mg/kg for 5 days; total RNA was extracted from PC-3 tumors 6 days after the initiation of treatment, and TRPM-2 and GAPDH mRNA levels were analyzed by Northern blotting. Lanes 1–3, PC-3 tumors in mice administered MM Co. ODN; Lanes 4–6, PC-3 tumors in mice administered AS ODN #2. B, quantitative analysis of TRPM-2 mRNA levels after normalization to GAPDH mRNA levels in PC-3 tumors after treatment with AS ODN #2 or MM Co. ODN was performed using a laser densitometer. Columns, means; bars, SD. *, differs from control (P < 0.01) by Student's t test.

length × width × depth × 0.5236 (33). Data points were reported as average tumor volumes ± SD.

TUNEL Staining. A modified TUNEL technique (34) was used to detect apoptotic cells in PC-3 tumors using the ApopTag In situ Apoptosis Detection System (Oncor, Gaithersburg, MD), according to the manufacturer's protocol. The number of positively stained cells/high power field in five random fields was counted and averaged.

Results

Screening for Active AS ODN Sequences Targeting the Human TRPM-2 Gene in PC-3 Cells. To identify effective AS ODNs capable of inhibiting TRPM-2 gene expression in PC-3 cells, 10 phosphorothioate ODNs designed to hybridize with various regions of TRPM-2 mRNA were synthesized (Fig. 1A). Northern blot analyses were used to evaluate the effects of treatment with these AS ODNs on TRPM-2 mRNA expression in PC-3 cells. As shown in Fig. 1, B and C, after daily treatment of PC-3 cells with 1 μM AS TRPM-2 ODN for 2 days, the different ODNs exhibited varied degrees of activity. Seven AS ODNs had little or no effect on TRPM-2 mRNA expression levels, whereas three ODNs had moderate effects. The most potent AS sODN identified from this series was AS ODN#2 (5'-CAGCAGCAGAGTCTTCATCAT-3'), which targets the human TRPM-2 translation initiation site, reducing TRPM-2

expression levels by 80% compared with MM Co. ODN treatment. AS ODN#2 was used in all subsequent experiments.

Sequence-specific and Dose-dependent Inhibition of TRPM-2 Expression by AS TRPM-2 ODN. To further define the specificity and potency of AS ODN#2-mediated inhibition of TRPM-2 gene expression, the effects of AS ODN#2 and MM Co. ODN on TRPM-2 mRNA and protein levels were determined by Northern and Western blot analyses, respectively. As shown in Fig. 2, A and B, daily treatment of PC-3 cells with AS ODN#2 (100, 500, or 1000 nM) for 2 days reduced TRPM-2 mRNA levels by 17, 39, or 78%, respectively, whereas TRPM-2 mRNA expression was not affected by the MM Co. ODN at any of the used concentrations. Inhibition of TRPM-2 protein levels in PC-3 cells was also observed after daily treatment with AS ODN#2 for 4 consecutive days (Fig. 2C).

Effects of AS TRPM-2 ODN Treatment on the Growth of PC-3 Cells Both in Vitro and in Vivo. To determine whether the reduction of TRPM-2 expression affects the growth of PC-3 cells in vitro, the growth rates of PC-3 cells after treatment with various concentrations of AS ODN#2 or MM Co. ODN once daily for 2 days were examined using the MTT assay. No significant difference in PC-3 cell growth was observed between AS ODN#2 and MM Co. ODN treatment (data not shown).

We then evaluated the effects of AS ODN#2 treatment on the growth of PC-3 tumors in vivo. Male nude mice bearing PC-3 tumors ~1 cm in diameter were randomly selected for treatment with AS ODN#2 versus MM Co. ODN, and 10 mg/kg ODN was administered once daily by i.p. injection for 28 days. As shown in Fig. 3, there was no significant difference in PC-3 tumor growth between these two groups.

To determine whether AS ODN#2 treatment inhibits TRPM-2 expression in PC-3 tumors in vivo, each of three tumor-bearing nude mice were given 10 mg/kg AS ODN#2 or MM Co. ODN i.p. once daily for 5 days, and TRPM-2 mRNA expression levels in harvested tumor tissues were then analyzed by Northern blotting. Treatment with AS ODN#2 resulted in a 68% reduction in TRPM-2 mRNA levels in PC-3 tumors compared with MM Co. ODN-treated tumors (Fig. 4, A and B).

Enhanced Chemosensitivity of PC-3 Cells in Vitro with AS TRPM-2 ODN Treatment. To determine whether treatment with AS ODN#2 enhances the cytotoxic effects of paclitaxel and mitoxantrone, PC-3 cells were treated with 500 nM AS ODN#2 or MM Co. ODN once daily for 2 days and then incubated with medium containing various concentrations of either paclitaxel or mitoxantrone for 2 days. The MTT assay was then performed to determine cell viability. As shown in Fig. 5, A and B, AS ODN#2 treatment significantly enhanced chemosensitivity of paclitaxel and mitoxantrone in a dose-dependent manner, reducing the IC50 of paclitaxel and mitoxantrone by more than 60 and 50%, respectively.

The DNA fragmentation assay was performed to compare induction of apoptosis after treatment with 500 nM AS ODN#2 either alone or in combination with 5 nM paclitaxel or 1 nM mitoxantrone. Using the same treatment schedule described above, the characteristic apoptotic DNA ladder was observed only with combined treatment of AS ODN#2 plus paclitaxel or mitoxantrone (Fig. 5C). We further evaluated the effects of combined AS ODN#2 plus chemotherapy by using Western blot
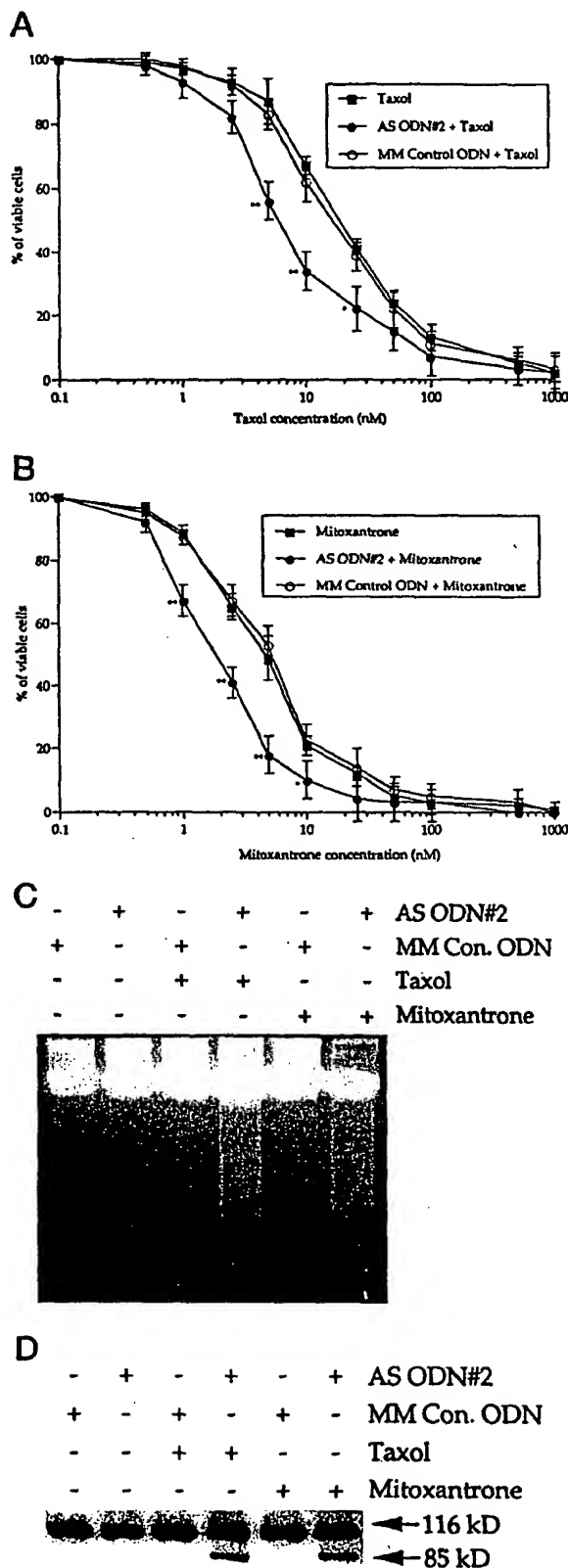
analysis to identify cleavage of PARP protein, a substrate of the caspases activated during the process of apoptotic execution (35). The M_r 116,000 intact form of PARP was observed in all samples examined, whereas the M_r 85,000 PARP cleavage fragment was detected only after combined treatment with AS ODN#2 plus paclitaxel or mitoxantrone (Fig. 5D).

Enhanced Cytotoxic Effects of Chemotherapy in PC-3 Tumors *in Vivo* by Systemic Administration of AS TRPM-2 ODN. Athymic male mice bearing PC-3 tumors ~1 cm in diameter were randomly selected for treatment with AS ODN#2 plus paclitaxel, MM Co. ODN plus paclitaxel, AS ODN#2 plus mitoxantrone, or MM Co. ODN plus mitoxantrone. Mean tumor volume was similar at the beginning of treatment in each of these groups. After randomization, 10 mg/kg AS ODN#2 or MM Co. ODN were injected i.p. once daily for 28 days. From days 10 to 14 and from days 24 to 28, 0.5 mg polymeric micellar paclitaxel or 0.3 mg mitoxantrone was administered once daily by i.v. injection. As shown in Fig. 6, A and B, AS ODN#2 significantly enhanced the apoptotic effects of micellar paclitaxel and mitoxantrone in PC-3 tumors, reducing mean tumor volume by more than 80 and 60%, respectively, by 8 weeks after initiation of treatment. In addition, TUNEL staining detected a 5- or 3-fold increase in the numbers of apoptotic cells in the PC-3 tumors treated with AS ODN#2 plus micellar paclitaxel or mitoxantrone, respectively, compared with those treated with MM Co. ODN plus micellar paclitaxel or mitoxantrone. Under the experimental conditions used in the above *in vivo* experiments, no side effects associated with ODN treatment and/or chemotherapy were observed.

Discussion

In the prostate gland, despite the original hypothesis that TRPM-2 is a marker of programmed cell death (12, 23–25), several experimental and clinical studies have provided conflicting findings showing the dissociation of TRPM-2 expression from apoptosis (26–30). For example, increased TRPM-2 expression in dysplastic lesions of rat prostate was not associated with enhanced apoptotic activity (29). Introduction of the *TRPM-2* gene into LNCaP prostate cancer cells renders them

Fig. 5 Effect of combined treatment with AS TRPM-2 ODN and chemotherapy on PC-3 cell growth and apoptosis. *A*, PC-3 cells were treated daily with 500 nm AS ODN#2 or MM Co. ODN for 2 days. After ODN treatment, the medium was replaced with medium containing various concentrations of Taxol (paclitaxel). After 48 h of incubation, cell viability was determined by the MTT assay. *Points*, means of triplicate analyses; *bars*, SD. ** and *, differ from control ($P < 0.01$ and $P < 0.05$, respectively) by Student's *t* test. *B*, PC-3 cells were treated daily with 500 nm AS ODN#2 or MM Co. ODN for 2 days. After ODN treatment, the medium was replaced with medium containing various concentrations of mitoxantrone. After 48 h of incubation, cell viability was determined by the MTT assay. *Points*, means of triplicate analyses; *bars*, SD. ** and *, differ from control ($P < 0.01$ and $P < 0.05$, respectively) by Student's *t* test. *C*, after the same treatment schedule as described *A* and *B*, DNA was extracted from PC-3 cells, electrophoresed in a 2% agarose gel, and visualized by ethidium bromide staining and UV transillumination. *D*, proteins were extracted from PC-3 cells after the same treatment as described in *A* and *B* and analyzed by Western blotting with an anti-PARP antibody. Uncleaved intact PARP, M_r 116,000; cleaved PARP, M_r 85,000.
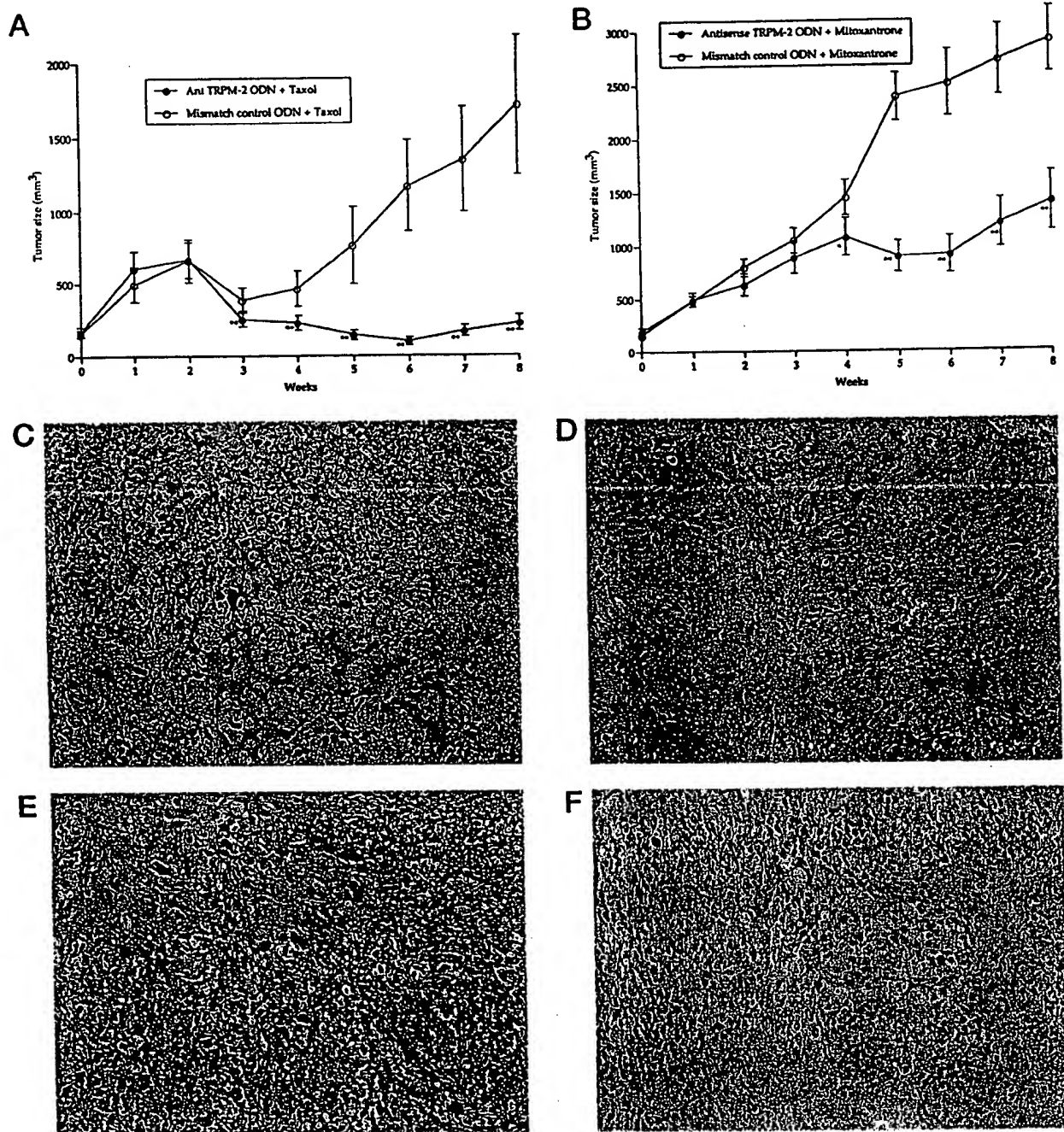
Fig. 6 Effects of combined treatment with AS TRPM-2 ODN plus chemotherapy on PC-3 tumor growth. *A* and *B*, mice bearing PC-3 tumors were randomly selected for treatment with AS ODN#2 plus micellar Taxol (paclitaxel) or MM Co. ODN plus micellar paclitaxel (*A*), or AS ODN#2 plus mitoxantrone or MM Co. ODN plus mitoxantrone (*B*). When PC-3 tumors became ~1 cm in diameter, 10 mg/kg AS ODN#2 or MM Co. ODN were daily injected i.p. for 28 days. From days 10 to 14 and from days 24 to 28, 0.5 mg micellar paclitaxel or 0.3 mg mitoxantrone was daily administered by i.v. injection. Tumor volume was measured once weekly and calculated by the formula: length × width × depth × 0.5236. *Points,* mean tumor volume in each experimental group containing eight mice; *bars,* SD. ** and *, differ from control ($P < 0.01$ and $P < 0.05$, respectively) by Student's *t* test. *C–F,* after completion of the same treatment schedule described in *A* and *B,* PC-3 tumors were harvested from each treatment group for detection of apoptosis using TUNEL staining. Sections of paraffin-embedded PC-3 tumors were stained with digoxigenin-dUTP antibody to identify apoptotic cells. *C,* PC-3 tumor after treatment with AS ODN#2 and micellar paclitaxel. *D,* PC-3 tumor after treatment with MM Co. ODN and micellar paclitaxel. *E,* PC-3 tumor after treatment with AS ODN#2 and mitoxantrone. *F,* PC-3 tumor after treatment with MM Co. ODN and mitoxantrone.

highly resistant to tumor necrosis factor-α-induced apoptosis (27). Furthermore, a close correlation between intracellular levels of TRPM-2 and tumor grade in human prostate cancer specimens has been reported (28). We also demonstrated previously that overexpression of TRPM-2 helps mediate AI progression against castration (31)- and chemotherapy[4]-induced apoptosis in androgen-dependent prostate cancer models. Collectively, these findings suggest that TRPM-2 up-regulation plays a protective role in normal and malignant prostate tissues against apoptosis induced by various kinds of stimuli and thereby may confer an aggressive phenotype during prostate cancer progression.

The limited efficacy of cytotoxic chemotherapy remains a major problem for the treatment of patients with advanced hormone refractory prostate cancer.

The lack of survival benefits with traditional cytotoxic chemotherapy in patients with hormone-refractory prostate cancer results from intrinsic chemoresistance and the limitation of toxicity on an elderly population. The chemoresistant phenotype in hormone-refractory prostate cancer is attributable, in part, to high levels of antiapoptotic genes, including Bcl-2, Bcl-xL, and TRPM-2, all of which are increased after androgen ablation and remain constitutively overexpressed in AI tumors (18, 36, 37).

Although no chemotherapeutic agent has demonstrated improved survival in patients with advanced prostate cancer, recent Phase II reports are documenting improved response rates in hormone-refractory disease (2, 36–38). For example, >50% of patients responded in one Phase II study of combined paclitaxel plus estramustine treatment (38). In two randomized trials, mitoxantrone in combination with prednisone was shown to produce significant palliative benefit compared with steroids alone (39, 40). Furthermore, recent preclinical studies have provided proof of principle evidence that targeting antiapoptotic genes using AS ODN enhances apoptosis induced by conventional cytotoxic chemotherapy (9–12). Therefore, in this study, we set out to screen for potent and sequence-specific ODNs targeted against human TRPM-2. The AS TRPM-2 ODN corresponding to the human TRPM-2 translation initiation site was the most potent sequence of the 10 AS ODN targeting various regions of TRPM-2 gene. To clarify the functional role of TRPM-2 expression in AI prostate cancer, we then tested the effects of AS TRPM-2 ODN on human prostate cancer PC-3 cell growth and whether AS TRPM-2 ODN could enhance the cytotoxic effects of paclitaxel and mitoxantrone in this model system.

Phosphorothioate AS TRPM-2 ODN used in this study significantly inhibited expression of TRPM-2 mRNA and protein in PC-3 cells both in vitro and in vivo. Sequence specificity was confirmed using a two-base TRPM-2 MM ODN, which had no effects on TRPM-2 expression in PC-3 cells. Despite a significant decrease in TRPM-2 expression after AS ODN treatment, no differences in PC-3 cell growth in vitro or PC-3 tumor growth in vivo was observed after AS TRPM-2 ODN treatment. These findings suggest that targeting and inhibiting TRPM-2 expression have no significant effects on cell proliferation in the absence of other apoptotic stimuli or cell death signals.

The administration of AS TRPM-2 ODN with chemotherapeutic agents, however, inhibited PC-3 cell growth both in vitro and in vivo through enhanced apoptosis. Pretreatment of PC-3 cells with AS enhanced apoptosis induced by these agents. Consistent with these in vitro studies, synergistic effects of combined use of AS TRPM-2 ODN plus chemotherapeutic agents were also observed in in vivo studies. Systemic administration of AS TRPM-2 ODN plus polymeric micellar paclitaxel or mitoxantrone suppressed the PC-3 tumor growth by 90 and 50%, respectively, compared with treatment with MM Co. ODN plus either agent. Detection of increased apoptotic cells after combined AS ODN and chemotherapy by TUNEL staining in PC-3 tumors suggests that decreased tumor progression rates after combined AS TRPM-2 ODN plus paclitaxel or mitoxantrone resulted from enhanced chemotherapy-induced apoptosis rather than decreased cell proliferation.

The results in the present study suggest that increased TRPM-2 helps mediate prostate cancer progression by inhibiting apoptotic cell death induced by several kinds of therapy, including cytotoxic chemotherapy. Decreasing TRPM-2-mediated chemoresistance by AS TRPM-2 ODN may provide a feasible and safe strategy to enhance chemosensitivity in hormone-refractory prostate cancer. The preclinical data presented here provide proof of principle support for designing clinical studies with combined AS TRPM-2 plus paclitaxel and/or mitoxantrone therapy for patients with hormone-refractory disease.

References

1. Denis, L., and Murphy, G. P. Overview of Phase III trials on combined androgen treatment in patients with metastatic prostate cancer. Cancer (Phila.), 72: 3888–3895, 1993.

2. Oh, W. K., and Kantoff, P. W. Management of hormone refractory prostate cancer: current standards and future prospects. J. Urol., 160: 1220–1229, 1998.

3. Crooke, S. T. Therapeutic applications of oligonucleotides. Annu. Rev. Pharmacol. Toxicol., 32: 329–376, 1992.

4. Wagner, R. W. The state of the art in antisense research. Nat. Med., 1: 1116–1118, 1995.

5. Monia, B. P., Johnston, J. F., Geiger, T., Muller, M., and Fabbro, D. Antitumor activity of a phosphorothioate antisense oligodeoxynucleotide targeted against c-raf kinase. Nat. Med., 2: 668–675, 1996.

6. Cucco, C., and Calabretta, B. In vitro and in vivo reversal of multidrug resistance in a human leukemia-resistant cell line by mdr1 antisense oligodeoxynucleotides. Cancer Res., 56: 4332–4337, 1996.

7. Ziegler, A., Luedke, G. H., Fabbro, D., Altman, K. H., Stahel, R. A., and Zangemeister-Wittke, U. Induction of apoptosis in small-cell lung cancer cells by an antisense oligodeoxynucleotide targeting the Bcl-2 coding sequence. J. Natl. Cancer Inst., 89: 1027–1036, 1997.

8. Miyake, H., Tolcher, A., and Gleave, M. E. Antisense Bcl-2 oligodeoxynucleotides inhibit progression to androgen-independence after castration in the Shionogi tumor model. Cancer Res., 59: 4030–4034, 1999.

9. Geiger, T., Muller, M., Monia, B. P., and Fabbro, D. Antitumor activity of a c-raf antisense oligodeoxynucleotide in combination with standard chemotherapeutic agents against various human tumors transplanted subcutaneously into nude mice. Clin. Cancer Res., 3: 1179–1185, 1997.

10. Jansen, B., Schlagbauer-Wadl, H., Brown, B. D., Bryan, R. N., van Elisas, A., Muller, M., Wolff, K., Eichler, H. G., and Pehamberger, H. bcl-2 antisense therapy chemosensitizes human melanoma in SCID mice. Nat. Med., 4: 232–234, 1998.

11. Campbell, M. J., Dawson, M., and Koeffler, H. P. Growth inhibition of DU-145 prostate cancer cells by a Bcl-2 antisense oligodeoxynucleotide is enhanced by N-(2-hydroxyphenyl)all-trans retinamide. Br. J. Cancer, 77: 739–744, 1998.

12. Miyake, H., Tolcher, A., and Gleave, M. E. Chemosensitization and delayed androgen-independent recurrence of prostate cancer with the use of antisense Bcl-2 oligodeoxynucleotides. J. Natl. Cancer Inst., 92: 34–41, 2000.

13. Blaschuk, O., Burdzy, K., and Fritz, I. B. Purification and characterization of a cell-aggregating factor (clusterin), the major glycoprotein in ram rete testis fluid. J. Biol. Chem., 258: 7714–7720, 1983.

14. Rosenberg, M. E., and Silkensen, J. Clusterin: physiologic and pathophysiologic considerations. Int. J. Biochem. Cell. Biol., 27: 633–645, 1995.

15. Connor, J., Buttyan, R., Olsson. C. A., D'Agati, V., O'Toole, K., and Sawczuk, I. S. SGP-2 expression as a genetic marker of progressive cellular pathology in experimental hydronephrosis. Kidney Int., 39: 1098–1103, 1991.

16. May, P. C., Lampert-Etchells, M., Johnson, S. A., Poirier, J., Masters, J. N., and Finch, C. E. Dynamics of gene expression for a hippocampal glycoprotein elevated in Alzheimer's disease and in response to experimental lesions in rat. Neuron, 5: 831–839, 1990.

17. Kyprianou, N., English, H. F., Davidson, N. E., and Isaacs, J. T. Programmed cell death during regression of the MCF-7 human breast cancer following estrogen ablation. Cancer Res., 51: 162–166, 1991.

18. Wunsche, W., Tenniswood, M. P., Schneider, M. R., and Vollmer, G. Estrogenic regulation of clusterin mRNA in normal and malignant endometrial tissue. Int. J. Cancer, 76: 684–688, 1998.

19. Schwochau, G. B., Nath, K. A., and Rosenberg, M. E. Clusterin protects against oxidative stress in vitro through aggregative and non-aggregative properties. Kidney Int., 53: 1647–1653, 1998.

20. French, L. E., Sappino, A. P., Tschopp, J., and Schifferli, J. A. Distinct sites of production and deposition of the putative cell death marker clusterin in the human thymus. J. Clin. Investig., 90: 1919–1925, 1992.

21. French, L. E., Wohlwend, A., Sappio, A. P., Tschopp, J., and Schifferli, J. A. Human clusterin gene expression is confined to surviving cells during in vitro programmed cell death. J. Clin. Investig., 93: 877–884, 1994.

22. Montpetit, M. L., Lawless, K. R., and Tenniswood, M. Androgen-repressed messages in the rat ventral prostate. Prostate, 8: 25–36, 1986.

23. Sensibar, J. A., Griswold, M. D., Sylvester, S. R., Buttyan, R., Bardin, C. W., Cheng, C. Y., Dudek, S., and Lee, C. Prostatic ductal system in rats: regional variation in localization of an androgen-repressed gene product, sulfated glycoprotein-2. Endocrinology, 128: 2091–2102, 1991.

24. Kyprianou, N., English, H. F., and Isaacs, J. T. Programmed cell death during regression of PC-82 human prostate cancer following androgen ablation. Cancer Res., 50: 3748–3753, 1990.

25. Wright, P. S., Cross-Doersen, D., Th'ng, J. P., Guo, X. W., Crissman, H. A., Bradbury, E. M., Montgomery, L. R., Thompson, F. Y., Loudy, D. E., Johnston, J. O., and Bitonti, A. J. A ribonucleotide reductase inhibitor, MDL 101,731, induces apoptosis and elevates TRPM-2 mRNA levels in human prostate tumor xenografts. Exp. Cell Res., 222: 54–60, 1996.

26. Brandstrom, A., Westin, P., Bergh, A., Cajander, S., and Damber, J. E. Castration induces apoptosis in the ventral prostate but not in an androgen-sensitive prostatic adenocarcinoma in the rat. Cancer Res., 54: 3594–3601, 1994.

27. Sensibar, J. A., Sutkowski, D. M., Raffo, A., Buttyan, R., Griswold, M. D., Sylvester, S. R., Kozlowski, J. M., and Lee, C. Prevention of cell death induced by tumor necrosis factor α in LNCaP cells by overexpression of sulfated glycoprotein-2 (clusterin). Cancer Res., 55: 2431–2437, 1995.

28. Steinberg, J., Oyasu, R., Lang, S., Sintich, S., Rademaker, A., Lee, C., Kozlowski, J. M., and Sensibar, J. A. Intracellular levels of SGP-2 (clusterin) correlate with tumor grade in prostate cancer. Clin. Cancer Res., 3: 1701–1711, 1997.

29. Ho, S. M., Leav, I., Ghatak, S., Merk, F., Jagannathan, V. S., and Mallery, K. Lack of association between enhanced TRPM-2/clusterin expression and increased apoptotic activity in sex-hormone-induced prostatic dysplasia of the Noble rat. Am. J. Pathol., 153: 131–139, 1998.

30. Sintich, S. M., Steinberg, J., Kozlowski, J. M., Lee, C., Pruden, S., Sayeed, S., and Sensibar, J. A. Cytotoxic sensitivity to tumor necrosis factor-α in PC3 and LNCaP prostatic cancer cells is regulated by extracellular levels of SGP-2 (clusterin). Prostate, 39: 87–93, 1999.

31. Miyake, H., Nelson, C., Rennie, P. S., and Gleave, M. E. Testosterone-repressed prostate message-2 is an antiapoptotic gene involved in progression to androgen independence in prostate cancer. Cancer Res., 60: 170–176, 2000.

32. Miyake, H., Hanada, N., Nakamura, H., Kagawa, S., Fujiwara, T., Hara, I., Eto, H., Gohji, K., Arakawa, S., Kamidono, S., and Saya, H. Overexpression of Bcl-2 in bladder cancer cells inhibits apoptosis induced by cisplatin and adenoviral-mediated p53 gene transfer. Oncogene, 16: 933–943, 1998.

33. Gleave, M., Hsieh, J. T., Gao, C. A., von Eschenbach, A. C., and Chung, L. W. Acceleration of human prostate carcinoma growth in vivo by factors produced by prostate and bone fibroblasts. Cancer Res., 51: 3753–3761, 1991.

34. Gavrieli, Y., Sherman, Y., and Ben-Sasson, S. A. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. J. Cell Biol., 119: 493–501, 1992.

35. Lazebnik, Y. A., Kaufmann, S. H., Desnoyers, S., Poirier, G. G., and Earnshaw, W. C. Cleavage of poly(ADP-ribose) polymerase by a proteinase with properties like ICE. Nature (Lond.), 371: 346–347, 1994.

36. Bruchovsky, N., Snoek, R., Rennie, P., Akakura, K., Goldenberg, S. L., and Gleave, M. E. Control of tumor progression by maintenance of apoptosis. Prostate, 6: 13–21, 1996.

37. Gleave, M., Tolcher, A., Miyake, H., Nelson, C., Brown, B., Beraldi, E., and Goldi, J. Progression to androgen independence is delayed by adjuvant treatment with antisense Bcl-2 oligodeoxynucleotides after castration in the LNCaP prostate tumor model. Clin. Cancer Res., 5: 2891–2898, 1999.

38. Hudes, G. R., Nathan, F. E., Khater, C., Greenberg, R., Gomella, L., Stern, C., and McAleer, C. Paclitaxel plus estramustine in metastatic hormone-refractory prostate cancer. Semin. Oncol., 22: 41–45, 1995.

39. Tannock, I. F., Osoba, D., Stockler, M. R., Ernst, D. S., Neville, A. J., Moore, M. J., Armitage, G. R., Wilson, J. J., Venner, P. M., Coppin, C. M., and Murphy, K. C. Chemotherapy with mitoxantrone plus prednisone or prednisone alone for symptomatic hormone-resistant prostate cancer: a Canadian randomized trial with palliative end points. J. Clin. Oncol., 14: 1756–1762, 1996.

40. Kantoff, P. W., Halabi, S., Conaway, M., Picus, J., Kirshner, J., Hars, V., Trump, D., Winer, E. P., and Vogelzang, N. J. Hydrocortisone with or without mitoxantrone in men with hormone-refractory prostate cancer: results of the cancer and leukemia group B 9182 study. J. Clin. Oncol., 17: 2506–2513, 1999.
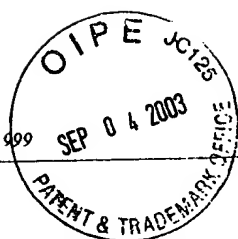
c-*raf*-1 Depletion and Tumor Responses in Patients Treated with the c-*raf*-1 Antisense Oligodeoxynucleotide ISIS 5132 (CGP 69846A)[1]

Peter J. O'Dwyer,[2] James P. Stevenson,
Maryann Gallagher, Amy Cassella,
Irina Vasilevskaya, Brett P. Monia,
Jon Holmlund, F. Andrew Dorr, and
Kang-Shen Yao

Thomas Jefferson University, Kimmel Cancer Center, Philadelphia, Pennsylvania 19104 [P. J. O., J. P. S., M. G., A. C., I. V., K-S. Y.], and ISIS Pharmaceuticals, Inc., Carlsbad, California [B. P. M., J. H., F. A. D.]

ABSTRACT

Abnormally regulated signaling through proliferative signal transduction pathways characterizes many of the common solid tumors. The best described of these involves potentially oncogenic proteins of the Ras family, which activate Raf proteins in the early steps of the mitogen-activated protein kinase cascade. ISIS 5132, a phosphorothioate antisense oligodexoynucleotide directed to the 3' untranslated region of the c-*raf*-1 mRNA, inhibits the growth of human tumor cell lines *in vitro* and *in vivo* in association with specific down-regulation of target message expression. Using a semiquantitative reverse transcription-PCR assay, we analyzed changes in c-*raf*-1 mRNA expression in peripheral blood mononuclear cells collected from patients with advanced cancers treated with ISIS 5132 as part of a clinical trial. Specimens were collected for analysis pretreatment and on days 3, 5, 8, and 15 of the first cycle and on day 1 of each subsequent cycle. We observed significant reductions of c-*raf*-1 expression from baseline by day 3 in 13 of 14 patients ($P = 0.002$). The time course and depletion of c-*raf*-1 message in peripheral blood mononuclear cells paralleled the clinical benefit in two patients. These findings demonstrate that ISIS 5132 specifically reduces target gene expression in treated patients and that peripheral blood mononuclear cells are suitable tissues for biomarker studies in future trials.

INTRODUCTION

The elucidation of oncogenic intracellular signaling pathways provides novel specific targets for antineoplastic intervention. The signaling molecule c-*raf*-1 is one of three highly conserved members of the *raf* gene family, which code for a group of serine/threonine protein kinases best known for their role in growth factor receptor-mediated signal transduction through the mitogen-activated protein kinase pathway (1, 2). After recruitment to the plasma membrane by activated Ras, Raf-1 is activated by phosphorylation and begins a sequence of downstream events including the phosphorylation of MEK1[3] and MEK2 that in turn activate ERK1 and ERK2 (3–5). Activated ERK1 and ERK2 then translocate to the nucleus, where they have multiple effects on gene expression mediated by the induction of transcription factors such as Elk-1 and Ets-2 and by activation of the cyclins D1 and E (2, 6, 7). The targets of activated ERK1 and ERK2 play key roles in cellular proliferation and differentiation (6). The transcription factor nuclear factor-κβ can be activated by Raf-1 in the cytoplasm and has additional transforming effects that are independent of the ERK pathway (8). Recent work has shown the antiapoptotic protein Bcl-2 to activate Raf-1 on outer mitochondrial membranes; here Raf-1 inhibits the inactivation of Bcl-2 by BAD, allowing Bcl-2-mediated suppression of apoptosis (9).

Mutations in *ras* genes have been reported in a range of solid tumors and leukemias, most notably in pancreatic adenocarcinomas in which >80% of patients have mutated K-*ras* (10). Mutated Ras is constitutively active, with cellular transformation resulting from Raf-1 activation of downstream effectors in the ERK1 and ERK2 pathway (11). However, the complexity of Raf-1 regulation extends beyond its interaction with Ras. Considerable evidence suggests that there are multiple Ras-independent activators of Raf-1, including the tyrosine kinases Src (12) and JAK1 (13), protein kinase C-α (14, 15), ceramide-activated protein kinase (16), as well as Raf-1 oligomerization (17). Raf-1 itself also possesses oncogenic potential; NH_2-terminal deletion mutants have transforming activity, and *raf* gene mutations have been detected in human tumors (18, 19). The establishment of Raf-1 as an important mediator of diverse signaling pathways underscores its involvement in malignant transformation and provides a rationale for Raf-directed novel therapies.

A 20-mer phosphorothioate antisense ODN targeted to the 3' untranslated region of c-*raf*-1 mRNA inhibited the growth of tumor cell lines *in vitro* at concentrations that effect sequence-specific depletion of c-*raf*-1 mRNA and protein (20, 21). Administration of this drug (ISIS 5132, CGP 69846A) to immunodeficient rodents bearing human tumor xenografts results in marked tumor growth delay (ISIS 5132 Investigator's Brochure,

[3] The abbreviations used are: MEK, MAP kinase kinase; ERK, extracellular-signal regulated kinase; ODN, oligodeoxynucleotide; PBMC, peripheral blood mononuclear cell; CT, computed tomography; RT-PCR, reverse transcription-PCR; CEA, carcinoembryonic antigen.

1998). Depletion of c-raf-1 mRNA was also observed in the tumors of treated animals.

We performed a clinical trial of ISIS 5132 to determine its toxicity profile and clinical effects in humans, to describe its pharmacokinetics, and as reported here to determine whether administration of the antisense construct at tolerable doses could exert a selective biological effect. The effect was measured in a surrogate tissue, the PBMC. We found that this treatment resulted in a pronounced depletion of c-raf-1 kinase mRNA in most of the patients treated in doses ≥2.5 mg/kg on a three times weekly schedule. In most of the patients, there was evidence of partial recovery of expression by day 15, despite continued treatment. In two patients, prolonged inhibition of tumor growth was observed in association with these effects.

These data demonstrate for the first time that the administration of antisense drugs directed to active oncogenic proliferative pathways may result in both depletion of the target mRNA and a therapeutic antitumor effect in patients.

PATIENTS AND METHODS

Patient Population. Patients treated in this study were at least 18 years of age and had histologically proven cancers that were refractory to standard therapies. All patients had an Eastern Cooperative Oncology Group performance status ≤2, a life expectancy ≥12 months, and were recovered from previous treatment. Eligibility required adequate bone marrow, renal, and liver function, and no prolongation of the prothrombin time or activated partial thromboplastin time. The study was performed at Thomas Jefferson University between April 1996 and January 1998 and was approved by an institutional ethics board. Patients provided written informed consent prior to treatment.

Drug Preparation and Administration. ISIS 5132 is the 19-sodium salt of a 20-base ODN (5'-TCCCGCCTGTGA-CATGCATT-3') with 19 internucleotide phosphorothioate linkages and was manufactured by Isis Pharmaceuticals, Inc. to Good Manufacturing Practice standards using solid phase supported synthesis, followed by orthogonal preparative chromatographic purification and lyophilization (ISIS 5132 Investigator's Brochure, 1998). ISIS 5132 was supplied as a sterile solution in 2- and 10-ml vials at a concentration of 10 mg/ml, protected from light, and stored at 2–8°C. Prior to administration, ISIS 5132 was diluted in normal saline to a total volume of 50 ml and then infused i.v. over 2 h. Patients were treated at nine different dose levels ranging from 0.5 to 6.0 mg/kg with no intrapatient dose escalation. One treatment cycle comprised ISIS 5132 administration as a 2-h i.v. infusion three times weekly for three consecutive weeks, followed by 1 week of no treatment. Toxicity during each cycle was assigned according to the WHO Toxicity Grading Scale. Patients had CT scans performed after every alternate course and were evaluated for tumor progression or response using standard criteria (22).

Measurement of c-raf-1 mRNA Expression in PBMCs. Patients at the 2.5-mg/kg dose level and above had blood sampling (5–10 ml) for c-raf-1 mRNA analysis at baseline and on days 3, 5, 8, and 15 of the first treatment cycle and day 1 of each cycle thereafter. PBMCs were isolated by Ficoll-Hypaque density centrifugation and stored at −70°C. Total RNA was isolated from PBMCs using Trizol reagent (Life Technologies,

Inc., Rockville, MD) according to the manufacturer's directions. c-raf-1 expression was then quantitated using RT-PCR, as originally described by Horikoshi et al. (23), and modified by O'Dwyer et al. (24). Briefly, 100 ng of total RNA was used for each cDNA reaction. Varying amounts of cDNA (0.1–10 μl) within the linear range of amplification were then used as a substrate for the PCR amplification of c-raf-1 and β-actin. c-raf-1 expression was normalized to that of the endogenous standard β-actin by calculating the ratio of the radiolabeled PCR products.

The c-raf-1 primer sequences were: Raf (1), 5'-TCA-GAGAAGCTCTGCTAAG-3'; and Raf (2), 5'-CAATGCACT-GGACACCTTA-3'. β-actin primer sequences were: BA (67), 5'-GCGGGAAATCGTGCGTGACATT-3'; and BA (68), 5'-GATGGAGTTGAAGGTAGTTTCGTG-3' as described (23). cDNA synthesis was carried out with 2 μg of total RNA using Ready-to-Go You-Prime First-Strand Bead kits (Pharmacia Biotech, Uppsala, Sweden). PCR was carried out in Ready-to-Go PCR Bead kits (Pharmacia Biotech, Uppsala, Sweden). Both sets of reactions were performed according to the manufacturer's instructions. The PCR reactions (25 μl total volume, containing 1–10 μl cDNA, 12.5 pmol of each of the c-raf-1 or β-actin primers, and 1 μCi [α-^{32}P]dCTP) were heated to 95°C for 5 min and then amplified for 36 cycles at 95°C for 1 min, 55°C for 1 min, and 72°C for 2 min. The products were loaded on 8% urea polyacrylamide gels. The gels were dried at 80°C for 1 h under vacuum and exposed to film for several hours at −80°C. The band corresponding to c-raf-1 was either cut out and subjected to liquid scintillation counting (patients 1–8) or measured by densitometric scanning (patients 9–14). Day-to-day coefficients of variation were 24% for c-raf-1 and 15% for β-actin. Mean values of c-raf-1 expression on days 3, 5, 8, and 15 of ISIS 5132 therapy during the first cycle were compared with pretreatment expression using the Wilcoxon signed rank test.

RESULTS

Patient characteristics and study treatment have been described in detail elsewhere (25). Briefly, 29 fully evaluable patients with a range of cancer types received ISIS 5132 as a 2-h infusion three times weekly for 3 weeks. After a 1-week treatment-free interval, dosing was resumed and maintained as long as the patient remained free of tumor progression or significant toxicity. Doses were escalated from 0.5 to 6.0 mg/kg in cohorts of three patients. The drug was well tolerated, and no patient required dose reduction. Two patients, one with renal and one with colon cancers, experienced prolonged inhibition of tumor growth lasting more than 8 months.

Variability in c-raf-1 Expression PBMCs were obtained from patients treated at doses of 2.5 mg/kg and above taken from patients treated at doses of 2.5 mg/kg and above immediately prior to treatment on days 1, 3, 5, 8, and 15 of the first cycle. Samples were obtained on subsequent courses in patients who continued on therapy. Because of the low abundance of the c-raf-1 message in PBMCs, mRNA quantitation was performed using a semiquantitative RT-PCR assay. A typical autoradiograph of c-raf-1 and of β-actin is shown (Fig. 1).

Baseline c-raf-1 expression did not relate to patient age, sex, tumor type, or performance status. Similarly, no association
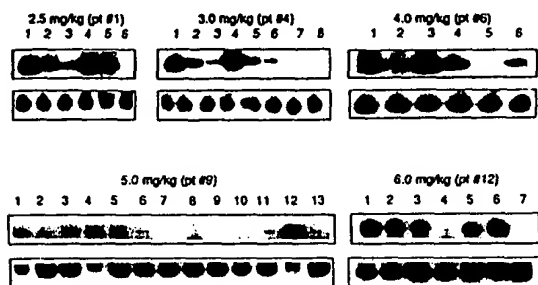
2.5 mg/kg (pt #1)   3.0 mg/kg (pt #4)   4.0 mg/kg (pt #6)
1 2 3 4 5 6   1 2 3 4 5 6 7 8   1 2 3 4 5 6

5.0 mg/kg (pt #9)   6.0 mg/kg (pt #12)
1 2 3 4 5 6 7 8 9 10 11 12 13   1 2 3 4 5 6 7

Fig. 1 RT-PCR analysis of c-*raf*-1 mRNA expression in PBMCs of five patients at each of the ISIS 5132 dose levels. Reduction of expression is noted in all five during cycle 1; this persisted during multiple cycles of therapy in patient 9 and corresponded to tumor growth inhibition. *Lanes 1-6* in each patient represent samples drawn on days 1, 3, 5, 8, 15, and 22 of cycle 1. *Lanes 7* and *8* from patient 9 represent samples drawn during subsequent cycles.

between c-*raf*-1 expression at baseline or during ISIS 5132 therapy and peripheral blood lymphocyte or monocyte counts was noted.

Depletion of c-*raf*-1 mRNA after Treatment. At all dose levels tested, depletion of c-*raf*-1 mRNA was observed within 48 h of the initial dose. Thirteen of 14 patients showed a decrease in message levels on day 3 ($P = 0.002$). No dose relationship was evident in this effect. The median decrease was to 42% (mean, 53%) of initial values (Table 1). When absolute values were considered, the mean mRNA content decreased from 1497 units at baseline to 602 units (40%) on day 3. Median values continued to be depleted to a median of 26% (mean, 71%) on day 5 ($P = 0.017$), with some evidence of recovery after 3 days without treatment on day 8 (median, 62%; mean, 81%; $P = 0.03$) and continued depletion on day 15 (median, 35%; mean, 74%; $P = 0.017$). The day at which the nadir was reached varied among patients; one reached nadir on day 3, seven on day 5, two on day 8, and four on day 15. The mean nadir value did not differ by dose of ISIS 5132, suggesting that all doses used exhibited similar biological effects (Table 1). Higher doses did not result in more protracted inhibition.

Biological Responses in Responding Patients. Two patients, both of whom had demonstrated tumor progression with previous cytotoxic chemotherapy, exhibited long-term stable disease in response to ISIS 5132 treatment. One was a 68-year-old man with colorectal cancer metastatic to liver who had progressed 2 years after adjuvant therapy with 5-fluorouracil/leucovorin and had evinced further tumor growth during therapy with a 17-1A monoclonal antibody and irinotecan. After treatment with 3 mg/kg of ISIS 5132, minor (20%) shrinkage in a liver metastasis was accompanied by a progressive decline in CEA from 895 to 618 ng/ml. During this time, c-*raf*-1 mRNA values declined to <10% of the initial value (Fig. 2). After seven cycles of treatment, both the plasma CEA values and the PBMC c-*raf*-1 mRNA began to increase, and 1 month later, the CT scan revealed progression of the hepatic metastases.

A 46-year-old woman with renal cell cancer metastatic to lung and lymph nodes failed to respond to interleukin-2, IFN-α, and 5-fluorouracil in combination and began treatment with

ISIS 5132 at 5 mg/kg. She had immediate symptomatic improvement, but the size of the tumor was unchanged on CT scans. After 10 cycles of treatment, she began to have recurrent pain, and progression was identified radiologically. In this patient, the nadir PBMC c-*raf*-1 mRNA was 12%, and values remained low until the beginning of the ninth cycle, when a return above baseline was observed, again followed shortly thereafter by progressive disease.

DISCUSSION

Inhibition of proliferative pathways has long been a goal of anticancer therapy, but progress has been hampered by the limited specificity of small molecule inhibitors. The demonstration of dysregulation of the signal transduction pathways of growth signaling as an early and continuing abnormality of carcinogenesis has provided novel targets for the control of tumor growth. Several proliferative mechanisms have been shown to converge on the ras/raf/MEK/ERK signal transduction axis, including *ras* and *raf* mutation, overproduction of autocrine growth factors, and inactivation by mutation of parallel growth inhibitory pathways (*e.g.*, transforming growth factor β; Refs. 2, 19, and 26). As the proximate membrane-associated protein in this pathway, Raf-1 is therefore a logical target.

The phosphorothioate antisense drug ISIS 5132 is targeted to a 20-base sequence of the 3′ untranslated region of c-*raf*-1 mRNA, which was demonstrated in cell culture to result in maximal depletion of the target message (20). In mice bearing human tumor xenografts, inhibition of tumor growth was associated with selective depletion of both mRNA and protein (21). A similar phosphorothioate drug targeted to the murine c-*raf*-1 gene was associated with minimal toxicity at doses that were effective in tumor growth inhibition (27). It was then incumbent upon the initial human studies to: (*a*) demonstrate that at dose levels associated with minimal toxicity a specific effect on c-*raf*-1 mRNA content could be achieved; and (*b*) suggest that the biological effect may result in some therapeutic benefit. Evidence is presented that both of these requirements have been met in a clinical trial.

As detailed fully elsewhere (25), the side effects associated with ISIS 5132 administration were mild and well tolerated. Fever and fatigue were the only symptoms, and laboratory abnormalities included anemia, short-lived elevation of the activated partial thromboplastin time, and transient dose-dependent elevation of the alternative pathway complement protein C3a. Dose escalation was halted at 6 mg/kg, because higher doses in simian models were associated with complement activation (28). None of the effects are believed to be specific to the antisense sequence, and similar constitutional symptoms have been reported with polynucleotides investigated in the 1970s and with other antisense constructs in early clinical trials (29–31).

To demonstrate an effect on c-*raf*-1 expression, we chose PBMCs as a surrogate tissue, based primarily on their availability and ease of repeated sampling, and their immediate exposure to ISIS 5132 in plasma. In previous studies, we have found that PBMC expression of certain detoxication genes correlates well with that in colon mucosa (25). An anticipated limitation was the known variability in antisense ODN uptake among various

Table 1 c-raf-1 mRNA expression in peripheral mononuclear cells of patients who had sampling performed during cycle 1 of ISIS 5132 treatment (expressed as a percentage of baseline activity)

Nadir expression percentages and day of nadir are represented in the final column. Values represent means of three replicates.

Dose	Patient no.	Baseline activity	Day 3 %	Day 5 %	Day 8 %	Day 15 %	Nadir, % (day)
2.5 mg/kg	1	2770	33	26	125	32	26 (5)
	2	2449	64	198	52	7	7 (15)
	3	2162	66	20	64	31	20 (5)
3 mg/kg	4	2253	22	8	111	17	8 (5)
	5	2118	12	23	46	85	12 (3)
4 mg/kg	6	2229	37	135	55	12	12 (15)
	7	1808	24	22	62	44	22 (5)
	8	1503	12	8	23	39	8 (5)
5 mg/kg	9	671	12	135	78	11	11 (15)
	10	841	47	NA[a]	131	0	0 (15)
	11	272	213	204	21	165	21 (8)
6 mg/kg	12	1008	72	55	6	106	6 (8)
	13	113	62	6	301	416	6 (5)
	14	764	65	41	62	99	41 (5)
Median %			42	26	62	36	
Mean %			53	68	81	76	

[a] NA, no sample available.
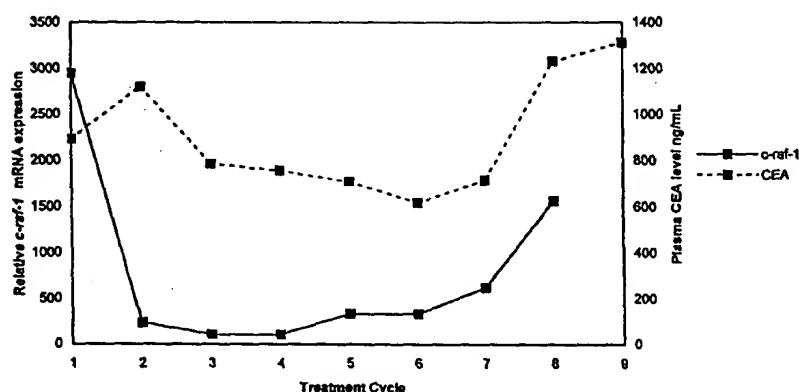
Fig. 2 Changes in plasma CEA level and c-raf-1 mRNA expression during ISIS 5132 treatment in a patient with colon cancer whose disease was inhibited for 8 months.

tissue types (32) and the recognized lower permeability of this cell type to antisense constructs in general (33). Therefore, the demonstration of a biological effect in this cell type is encouraging.

Almost all of the patients showed some evidence of downregulation of c-raf-1 in PBMCs. The effect was observed at all doses tested, and neither nadir values nor the duration of the effect varied with dose (Table 1). In six patients who demonstrated progressive depletion in the first week of therapy, evidence of recovery was observed in samples obtained on days 8 and 15. It is unclear if this pattern resulted from the latter samples being drawn 3 rather than 2 days after dosing (which may suggest recovery within 72 h of an individual dose), or if these findings may be indicative of an adaptive response. In the two patients who went on to receive multiple cycles of treatment, no such adaptive response was evident, but the possibility of generating an acquired resistant phenotype in certain individuals may reasonably be hypothesized. The basis for resistance to antisense therapy has not been described, but a parallel may exist with conventional cytotoxic agents (24, 34, 35). Down-

regulation of protein expression might also contribute to resistance. Benimetskaya et al. (36) have recently identified an antigen (Mac-1) on the surface of neutrophils that acts as a receptor that can mediate ODN internalization. Another potential source of resistance might be inferred from the response of the patient with colorectal cancer; despite continuing treatment, c-raf-1 mRNA levels in the PBMCs rose concomitantly with progression in plasma CEA values, followed shortly by CT scan evidence of tumor regrowth (Fig. 2). This time course may have a number of explanations, including a pharmacokinetic cause for the escape from inhibition (accelerated plasma clearance), or induction of resistance through alterations in cell signaling pathways.

The effects on c-raf-1 expression are suggestive of but do not prove an antisense effect. The observed time course is supportive, but the absence of specimens from the lower dose levels is unfortunate because all doses from 2.5 mg/kg demonstrated a biological effect. The difficulty of demonstrating an antisense effect has been emphasized by Stein (37), and guidelines for its analysis have been published (38, 39). Drug re-

sponses may be nonantisense but sequence selective (40–42) or nonantisense and sequence independent (43). The former type of response may occur with phosphorothioate antisense drugs bearing a CpG dinucleotide (as does ISIS 5132); stimulation of B and natural killer cells by these drugs has been reported (38). However, it has been demonstrated that ISIS 5132 does not stimulate natural killer activity, because it lacks CpG flanking sequences that are required for this effect (44). It seems unlikely, too, that an immune stimulatory effect would underlie the patient responses observed, especially in the patient with colorectal cancer, a tumor that is notably resistant to immunological modulation. Among the sequence-independent effects, it is recognized that binding of polyanionic antisense drugs to a variety, of peptides including some growth factors may occur. c-*raf*-1 expression at the mRNA level is independent of growth factor activity, however, and the very specific effect upon c-*raf*-1 mRNA levels in PBMCs supports a direct antisense mechanism, at least in this tissue.

These findings also raise the question of the optimal scheduling of ISIS 5132. Another Phase I trial using a 21-day continuous infusion has also reported antitumor effects (45). The broad range of dose-response using the short-term infusion suggests that an infusional schedule may achieve a more uniform biological and antitumor effect. Studies to address this issue are in progress.

REFERENCES

1. Daum, G., Eisenmann-Tappe, I., Fries, H. W., Troppmair, J., and Rapp, U. R. The ins and outs of Raf kinases. Trends Biol. Sci., *19:* 474–480, 1994.

2. Nishida, E., and Gotoh, Y. The MAP kinase cascade is essential for diverse signal transduction pathways. Trends Biol. Sci., *18:* 128–131, 1993.

3. Stokoe, D., MacDonald, S., Cadwallader, K., Symons, M., and Hancock, J. Activation of Raf as a result of recruitment to the plasma membrane. Science (Washington DC), *264:* 1463–1467, 1994.

4. Zhang, X. F., Settleman, J., Kyriakis, J. M., Takeuchi-Suzuki, E., Elledge, S. J., Marshall, M. S., Bruder, J. T., Rapp, U. R., and Avruch, J. Normal and oncogenic p21^{ras} proteins bind to the amino-terminal regulatory domain of c-Raf-1. Nature (Lond.), *364:* 308–313, 1993.

5. Cobb, M. H., and Goldsmith, E. J. How MAP kinases are regulated. J. Biol. Chem., *270:* 14843–14846, 1996.

6. McCormick, F. Ras-related proteins in signal transduction and growth control. Mol. Reprod. Dev., *42:* 500–506, 1995.

7. Lavoie, J. N., L'Allemain, G., Brunet, A., Muller, R., and Pouysse-gur, J. Cyclin D1 expression is regulated positively by the p42/44^{MAPK} and negatively by the p38/HOG^{MAPK} pathway. J. Biol. Chem., *271:* 20608–20616, 1996.

8. Li, S., and Sedivy, J. M. Raf-1 protein kinase activates the NF-κB transcription factor by dissociating the cytoplasmic NF-κB-IκB complex. Proc. Natl. Acad. Sci. USA, *90:* 9247–9251, 1993.

9. Wang, H-G., Rapp, U. R., and Reed, J. C. Bcl-2 targets the protein kinase Raf-1 to mitochondria. Cell, *87:* 629–638, 1996.

10. Bos, J. L. *ras* oncogenes in human cancer: a review. Cancer Res., *49:* 4682–4689, 1989.

11. Schaap, D., van der Wal, J., Howe, L. R., Marshall, C. J., and van Blitterswijk W. J. A dominant-negative mutant of *raf* blocks mitogen-activated protein kinase activation by growth factors and oncogenic p21^{ras}. J. Biol. Chem., *268:* 20232–20236, 1993.

12. Stokoe, D., and McCormick, F. Activation of c-Raf-1 by Ras and Src through different mechanisms: activation *in vivo* and *in vitro*. EMBO J., *16:* 2384–2396, 1997.

13. Stancato, L. F., Yu, C. R., Petricoin, E. F., III, and Larner, A. C. Beta interferon and oncostatin M activate raf-1 and mitogen-activated protein kinase through a JAK1-dependent pathway. Mol. Cell. Biol., *17:* 3833–3840, 1997.

14. Kolch, W., Heidecker, G., Kochs, G., Hummel, R., Vahidi, H., Mischak, H., Finkenzeller, G., Marme, D., and Rapp, U. R. Protein kinase Cα activates Raf-1 by direct phosphorylation. Nature (Lond.), *364:* 249–252, 1993.

15. Ueda, Y., Hirai, S., Osada, S., Suzuki, A., Mizuno, K., and Ohno, S. Protein kinase Cδ activates the MEK-ERK pathway in a manner independent of Ras and dependent on Raf. J. Biol. Chem., *271:* 23512–23519, 1996.

16. Yao, B., Zhang, Y., Delikat, S, Mathias, S., Basu, S., and Kolesnick, R. Phosphorylation of Raf by ceramide-activated protein kinase. Nature (Lond.), *378:* 307–310, 1995.

17. Farrar, M. A., Alberola-Ila, J., and Perlmutter, R. M. Activation of the Raf-1 kinase cascade by coumermycin-induced dimerization. Nature (Lond.), *383:* 178–181, 1996.

18. Stanton, V. P., Jr., and Cooper, G. M. Activation of human *raf* transforming genes by deletion of normal amino-terminal coding sequences. Mol. Cell. Biol., *7:* 1171–1179, 1987.

19. Storm, S. M., and Rapp, U. R. Oncogene activation: c-*raf*-1 gene mutations in experimental and naturally occurring tumors. Toxicol. Lett., *67:* 201–210, 1993.

20. Monia, B. P., Sasmor, H., Johnston, J. F., Freier, S. M., Lesnik, E. A., Muller, M., Geiger, T., Altmann, K. H., Moser, H., and Fabbro, D. Sequence-specific antitumor activity of a phosphorothioate oligodeoxyribonucleotide targeted to human C-*raf* kinase supports an antisense mechanism of action *in vivo*. Proc. Natl. Acad. Sci. USA, *93:* 15481–15484, 1996.

21. Monia, B. P., Johnston, J. F., Geiger, T., Muller, M., and Fabbro, D. Antitumor activity of a phosphorothioate antisense oligodeoxynucleotide targeted against c-*raf* kinase. Nat. Med., *2:* 668–675, 1996.

22. Miller, A. B., Hoogstraten, B., Staquet, M., and Winkler, A. Reporting results of cancer treatment. Cancer (Phila.), *47:* 207–214, 1981.

23. Horikoshi, T., Danenberg, K. D., Stadlbauer, T. H., Volkenandt, M., Shea, L. C., Aigner, K., Gustavsson, B., Leichman. L., Frosing, R., Ray, M., Gibson, N. W., Spears, C. P., and Danenberg, P. V. Quantitation of thymidylate synthase, dihydrofolate reductase, and DT-diaphorase gene expression in human tumors using the polymerase chain reaction. Cancer Res., *52:* 108–116, 1992.

24. O'Dwyer, P. J., Szarka, C. E., Yao, K. S., Halbherr, T. C., Pfeiffer, G. R., Green, F., Gallo, J. M., Brennan, J., Frucht, H., Goosenberg, E. B., Hamilton, T. C., Litwin, S., Balshem, A. M., Engstrom, P. F., and Clapper, M. L. Modulation of gene expression in subjects at risk for colorectal cancer by the chemopreventive dithiolethione oltipraz. J. Clin. Investig., *98:* 1210–1217, 1996.

25. Stevenson, J. P., Yao, K-S., Gallagher, M., Friedland, D., Mitchell, E. P., Cassella, A., Monia, B., Kwoh, T. J., Yu, R., Holmlund, J., Dorr, F. A., and O'Dwyer, P. J. Phase I clinical/pharmacokinetic and pharmacodynamic trial of the c-*raf*-1 antisense oligonucleotide ISIS 5132 (CGP 69846A). J. Clin. Oncol., *17:* 2227–2236, 1999.

26. Parsons, R., Myeroff, L. L., Liu, B., Willson, J. K., Markowitz, S. D., Kinzler, K. W., and Vogelstein B. Microsatellite instability and mutations of the transforming growth factor β type II receptor gene in colorectal cancer. Cancer Res., *55:* 5548–5550, 1995.

27. Henry, S. P., Monteith, D., and Levin, A. A. Antisense oligonucleotide inhibitors for the treatment of cancer. 2. Toxicological properties of phosphorothioate oligodeoxynucleotides. Anti-Cancer Drug Des., *12:* 395–408, 1997.

28. Henry, S. P., Monteith, D., Bennett, F., and Levin, A. A. Toxicological and pharmacokinetic properties of chemically modified antisense oligonucleotide inhibitors of PKC-α and c-*raf* kinase. Anti-Cancer Drug Des., *12:* 409–420, 1997.

29. Levine, S. A., Sivulich, M., Wiernik, P. H., and Levy, H. B. Initial clinical trials in cancer patients of polyriboinosinic-polyribocytidylic acid

stabilized with poly-L-lysine, in carboxymethylcellulose [poly(ICLC)], a highly effective interferon inducer. Cancer Res., *39:* 1645–1650, 1979.

30. Bishop, M. R., Iversen, P. L., Bayever, E., Sharp, J. G., Greiner, T. C., Copple, B. L., Ruddon, R., Zon, G., Spinolo, J., Arneson, M., Armitage, J. O., and Kessinger, A. Phase I trial of an antisense oligonucleotide OL(1)p53 in hematologic malignancies. J. Clin. Oncol., *14:* 1320–1326, 1996.

31. Glover, J. M., Leeds, J. M., Mant, T. G., Amin, D., Kisner, D. L., Zuckerman, J. E., Geary, R. S., Levin, A. A., and Shanahan, W. R., Jr. Phase I safety and pharmacokinetic profile of an intracellular adhesion molecule-1 antisense oligodeoxynucleotide (ISIS 2302). J. Pharmacol. Exp. Ther., *282:* 1173–1180, 1997.

32. Perez, J. R., Higgins-Sochaski, K. A., Maltese, J-Y., and Narayanan, R. Antisense *rel* A in cancer. *In:* S. Agrawal (ed.), Methods in Molecular Medicine: Antisense Therapeutics, pp. 183–199. Totowa: Humana Press, Inc., 1996.

33. Bargou, R. C., Jurchott, K., Wagener, C., Bergmann, S., Metzner, S., Bommert, K., Mapara, M. Y., Winzer, K. J., Dietel, M., Dorken, B., and Royer, H. D. Nuclear localization and increased levels of transcription factor YB-1 in primary human breast cancers are associated with intrinsic *MDR1* gene expression. Nat. Med., *4:* 447–450, 1997.

34. Geary, R. S., Leeds, J. M., Henry, S. P., Monteith, D. K., and Levin, A. A. Antisense oligonucleotide inhibitors for the treatment of cancer. 1. Pharmacokinetic properties of phosphorothioate oligodeoxynucleotides. Anti-Cancer Drug Des., *12:* 383–393, 1997.

35. Dumontet, C., Duran, G. E., Steger, K. A., Beketic-Oreskovic, L., and Sikic, B. I. Resistance mechanisms in human sarcoma mutants derived by single-step exposure to paclitaxel (Taxol). Cancer Res., *56:* 1091–1097, 1996.

36. Benimetskaya, L., Loike, J. D., Khaled, Z., Loike, G., Silverstein, S. C., Cao, L., el Khoury, J., Cai, T. Q., and Stein, C. A. Mac-1 (CD11b/CD18) is an oligodeoxynucleotide-binding protein. Nat. Med., *3:* 414–420, 1997.

37. Stein, C. A. Does antisense exist? Nat. Med., *1:* 1119–1121, 1995.

38. Stein, C. A., and Krieg, A. Problems in interpretation of data derived from *in vitro* and *in vivo* use of antisense oligodeoxynucleotides. Antisense Res. Dev., *4:* 67–69, 1994.

39. Crooke, S. T. Proof of mechanism of antisense drugs. Antisense Nucleic Acid Drug Dev., *6:* 145–147, 1996.

40. McIntyre, K. W., Lombard-Gillooly, K., Perez, J. R., Kunsch, C., Sarmiento, U. M., Larigan, J. D., Landreth, K. T., and Narayanan, R. A sense phosphorothioate oligonucleotide directed to the initiation codon of transcription factor NF-κB p65 causes sequence-specific immune stimulation. Antisense Res. Dev., *3:* 309–322, 1993.

41. Branda, R. F., Moore, A. L., Mathews, L., McCormack, J. J., and Zon, G. Immune stimulation by an antisense oligomer complementary to the *rev* gene of HIV-1. Biochem. Pharmacol., *45:* 2037–2043, 1993.

42. Mojcik, C. F., Gourley, M. F., Klinman, D. M., Krieg, A. M., Gmelig-Meyling, F., and Steinberg, A. D. Administration of a phosphorothioate oligonucleotide antisense to murine endogenous retroviral MCF *env* causes immune effects *in vivo* in a sequence-specific manner. Clin. Immunol. Immunopathol., *67:* 130–137, 1993.

43. Wagner, R. W. Gene inhibition using antisense oligodeoxynucleotides. Nature (Lond.), *372:* 333–335, 1994.

44. Boggs, R. T., McGraw, K., Condon, T., Flournoy, S., Villiet, P., Bennett, C. F., and Monia, B. P. Characterization and modulation of immune stimulation by modified oligonucleotides. Antisense Nucleic Acid Drug Dev., *7:* 461–471, 1997.

45. Holmlund, J., Nemunaitis, J., Schiller, J., Dorr, A., and Kisner, D. Phase I trial of c-*raf* antisense oligonucleotide ISIS 5132 (CGP 69846A) by 21-day continuous intravenous infusion (CIV) in patients with advanced cancer. Proc. Am. Soc. Clin. Oncol., *17:* 210a, 1998.

Efficacy of Treatment with Antisense Oligonucleotides Complementary to Immunoglobulin Sequences of bcl-2/Immunoglobulin Fusion Transcript in a t(14;18) Human Lymphoma-*scid* Mouse Model[1]

Mitchell R. Smith,[2] Tao Xie, Zhao-Zong Zhou, and Indira Joshi

Department of Medical Oncology, Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111

ABSTRACT

In t(14;18)-positive lymphoma cells, bcl-2 is expressed from a fusion mRNA transcript containing the full coding sequence of bcl-2 and 3' immunoglobulin sequences. We reported previously that antisense oligodeoxyribonucleotides directed at the bcl-2 translational start site, as well as those targeted to immunoglobulin sequences 3' of the translocation breakpoint, down-regulate bcl-2 and inhibit growth of the t(14;18)-positive lymphoma line WSU-FSCCL *in vitro*. We have developed a *scid* mouse model with this human cell line and demonstrate that antisense oligodeoxyribonucleotides targeted to immunoglobulin c_μ sequences down-regulate bcl-2 protein expression and induce apoptosis of WSU-FSCCL cells *in vivo*. This leads to prolonged survival of the mice. Targeting non-oncogenic sequences outside of the breakpoints of fusion transcripts may be a clinically useful therapeutic strategy.

INTRODUCTION

Specific genetic abnormalities represent targets for novel therapeutic strategies of malignancies. The most common genetic abnormality in non-Hodgkin's lymphoma is the chromosomal translocation t(14;18)(q32;q21) (1). From this translocation, a fusion transcript is expressed that contains the entire bcl-2 coding sequence with a 3' breakpoint fused to the immunoglobulin J_H region (1–4). Deregulated expression of bcl-2 prevents apoptosis and thus contributes to lymphoma develop-

ment. Antisense oligonucleotides targeted to bcl-2 sequences can down-regulate bcl-2 *in vitro* (5–10), *in vivo* in murine models (11–13), and in human trials (14). Although toxicity from bcl-2 down-regulation in normal tissues has not been observed as a major problem with short-term treatments in small numbers of patients (15), bcl-2 is expressed in a number of critical tissues (10, 16), and bcl-2 knockout mice have a range of abnormalities (15, 17). In attempting to design a specific method to down-regulate bcl-2 from the fusion transcript, we have targeted the immunoglobulin sequences fused to bcl-2 downstream of the breakpoint. We have reported (18) that AS[3] oligonucleotides designed to bind to these 3' immunoglobulin sequences specifically down-regulate bcl-2 expression and induce apoptosis in t(14;18)-containing WSU-FSCCL cells. We have developed a *scid* mouse model to further investigate the potential clinical utility of these immunoglobulin-targeted oligonucleotides against follicular lymphoma cells.

MATERIALS AND METHODS

Cell Line. WSU-FSCCL cells were grown as described (6) and resuspended in PBS at a concentration of 5×10^7 cells/ml, and 0.2 ml was injected/mouse. Routes of injection included i.p., via a tail vein, or s.c.

scid **Mice.** CB.17 *scid* mice were obtained from and housed in the Fox Chase Cancer Center Laboratory Animal Facility under an approved protocol. Mice used were females, 4–6 weeks of age, at the time of cell injection. Mice were checked daily by the laboratory animal facility staff and at least three times weekly by the investigators, per an approved animal use protocol. Mice were followed until death or, more usually, sacrificed by CO_2 inhalation when they appeared moribund or to be suffering.

Mice received injections of 1×10^7 FSCCL cells i.p. For survival experiments, repetitive doses of 200 μg (~10 mg/kg) of the indicated oligonucleotide were added at the times indicated. For assays of bcl-2 protein levels or apoptosis, lymphoma cells were allowed to grow for 5 weeks. At that time, 200–400 μg of the indicated oligonucleotide were injected i.p. Where indicated, the caspase inhibitor, Z-VAD (Enzyme Systems Products, Livermore, CA) was injected i.p. at a dose of 500 mg/mouse.

[3] The abbreviations used are: AS, antisense; *scid*, severe combined immunodeficient; Z-VAD, Z-Val-Ala-Asp; PE, phycoerythrin.

Flow Cytometry. Ascites or single-cell suspensions of spleen were suspended in PBSF (PBS containing 2.5% fetal bovine serum and 0.01% sodium azide). These were pelleted, washed once with PBS + 0.01% sodium azide, and then resuspended in the same solution. Cells in 100 μl were incubated with 20 μl of R-PE-conjugated antibody to CD38 or CD45 or to the negative control R-PE-IgG (PharMingen, San Diego, CA) for 30 min in the dark at 4°C. Cells were then washed in PBSF and once in PBS + 0.01% sodium azide. Cells were then fixed and permeabilized in 500 μl of 1.0% paraformaldehyde and 0.1% saponin (Sigma Chemical Co., St. Louis, MO) in the dark for 15 min at room temperature and washed twice in PBS + 0.1% sodium azide. Fifty μl of protein block serum-free (DAKO, Carpinteria, CA) and 50 μl of 0.1% saponin in PBS-azide were added to the pellet, gently vortexed, and incubated with 10 μl of FITC-anti bcl-2 antibody or FITC-conjugated negative control IgG antibody (DAKO) for 30 min in the dark at 4°C. Cells were washed twice in PBS-azide and resuspended in 300 μl of PBS-azide. Analysis was on a FACScan (Becton Dickinson).

Apoptosis. Apoptosis was assayed by use of APO 2.7 (Coulter). Cells were collected in PBS, washed in PBSF, and resuspended in 100 μl of cold PBSF + 0.1% digitonin (Sigma) for 20 min on ice. Cells were then washed in PBSF and pelleted. The cell pellet was resuspended in 80 μl of Apo 2.7-labeled with PE-Cy5 or control PE-IgG for 15 min at room temperature. Cells were washed in PBSF, resuspended in 0.5 ml of PBSF, and analyzed on a FACScan.

Western Blot. Single-cell suspensions of spleen or ascites were washed three times in PBS-azide. Cells were lysed at 30 μl of RIPA containing 100 μg/ml PMSF and 1 μg/ml aprotinin and incubated on ice for 30 min, followed by centrifugation at 1200 × g for 15 min at 4°C. The supernatant was removed and separated on 12% SDS-PAGE gels (Tris-HCl Ready-Gel; Bio-Rad, Hercules, CA). Transfer was to Immobilon-P membrane (Millipore, Bedford, MA), as suggested by the manufacturer. To the preblocked membrane, monoclonal mouse anti-bcl-2 antibody (clone bcl-2-100; Zymed Laboratories, Inc., South San Francisco, CA) was added at a 1:3000 dilution incubated for 1 h at room temperature. Antimouse IgG horseradish peroxidase (Amersham, Piscataway, NJ) at 1:2500 dilution was used as the secondary antibody for 30 min. Bands were detected by chemiluminescence (ECL) after exposing to Hyperfilm ECL (Amersham). Blots were stripped with 0.2 NaOH, blocked, and reprobed with unconjugated antibody to human CD38 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 1:1600 concentration for 1 h. Detection was as described above. Images from the X-ray films were scanned on a UMAX Vista-S6E scanner using V-scan 2.4.3 software on a Macintosh computer. The intensity of each band was analyzed by NIH image 1.61 software.

Oligonucleotides. Fully phosphorothioate-modified oligonucleotides were synthesized at the Fox Chase Cancer Center Macromolecular Core Facility. Sequences of oligonucleotides (5'-3') were: BCL-2 AS, gttctcccagcgtgcgc (19); BCL-2 mut, ttgcgcccctagggctc (6); c_μ AS, gaagacgctcactttgg (20); and c_μ mut, gtacaggcactgttagc. The mutated sequences have eight base changes that retain the same overall number of adenine, thy-

mine, guanine, and cytosine bases. Murine c_μ is: gaacacatttacattgg (21).

RESULTS

Establishment of the WSU-FSCCL-*scid* Model. For reproducible growth of WSU-FSCCL cells in *scid* mice, the mice were initially preconditioned with cyclophosphamide. Once the cells were passaged through the mice, however, they grew without such preconditioning. These cells could then be taken from the mice, maintained in cell culture, and still grow when reinjected into non-preconditioned *scid* mice. Whether cells were injected i.p., s.c., or i.v. did not alter the development of generalized lymphoma characterized by retroperitoneal and mesenteric adenopathy and infiltration of the liver, spleen, and marrow. Occasionally, a localized tumor developed after s.c. injection, but this was not observed consistently. After i.p. injection, ascites and mesenteric adenopathy were more prominent. Because discrete measurable tumors did not consistently develop with any of these methods of injection, survival was used as the endpoint for efficacy of treatment. For ease of administration, the i.p. route was used for these experiments.

After i.p. injection of 1×10^7 cells into CB.17 *scid* mice, 4–6 weeks of age, animals became visibly ill at 6–8 weeks and died or were euthanized at 8–11 weeks. At necropsy, the mice had lymphomatous ascites, splenomegaly, and bulky mesenteric and retroperitoneal adenopathy. Microscopically, spleen and marrow were replaced by lymphoma, whereas the liver revealed periportal infiltration with lymphoma. Kidneys were encased by, but not infiltrated with, lymphoma. Mice that survived longer because of treatment (see below) did develop meningeal involvement as well.

The lymphoma that developed *in vivo* closely recapitulated the characteristics of the cell line. The growth pattern was vaguely follicular. By flow cytometry, the lymphoma consisted of CD10+ B cells expressing CD19 and CD20. CD38 and CD45, but not CD5, CD23, or FMC7, were expressed. The major differences between the cell line *in vitro* and the lymphoma *in vivo* were that, in the mice, surface κ light chain became very faint, CD20 expression was less intense, and cell size was larger. By immunohistochemistry, bcl-2 and CD20 were positive.

Efficacy of c_μ Antisense Oligonucleotides. As an initial test of *in vivo* efficacy of AS oligonucleotides targeted to the immunoglobulin portion of the bcl-2-immunoglobulin fusion transcript, we examined ascites before and after i.p. injections of the oligonucleotides. Five weeks after injection, human cells were readily detectable in ascites fluid. These cells were demonstrated by dual staining using antihuman CD38 or CD45 and antihuman bcl-2. At this time, mice were treated with 200 μg (10 mg/kg) c_μ-AS oligonucleotide or mutated control (Fig. 1). By 2 days after c_μ-AS injection, rare human cells remained. Human cells began to reappear by 4 days and had returned to baseline numbers by 1 week after a single injection of c_μ-AS. No change was seen after control oligonucleotide injection.

Induction of Apoptosis by c_μ Antisense Oligonucleotides. Down-regulation of the antiapoptotic bcl-2 protein is predicted to induce apoptosis. Mice inoculated with WSU-FSCCL cells 5 weeks previously were treated with 200 μg (10

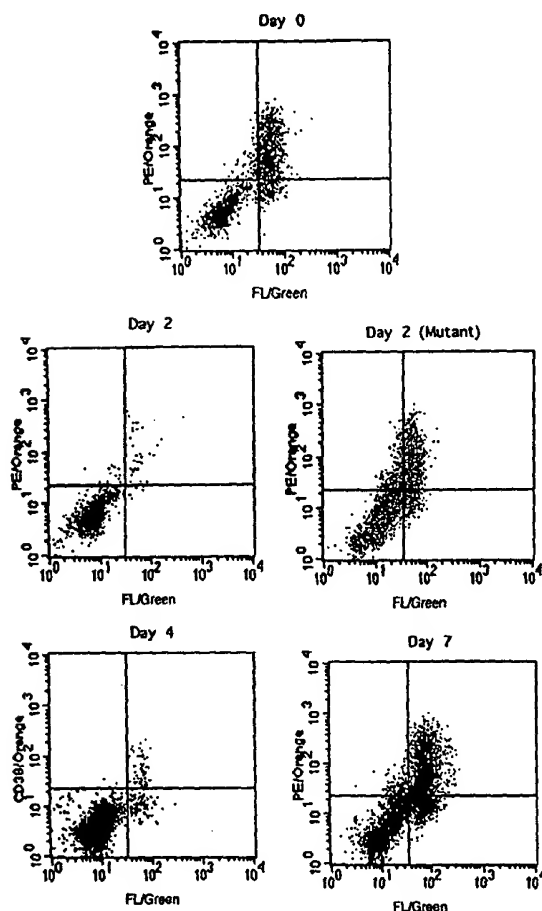**Day 0**

Day 2 **Day 2 (Mutant)**

Day 4 **Day 7**

Fig. 1 Flow cytometric analysis of ascites. WSU-FSCCL cells were injected i.p. and allowed to grow for 5 weeks. AS-c$_\mu$ oligonucleotides or control (*Mutant*) oligonucleotides (200 μg) were injected i.p., and ascites were removed on day 0, 2, 4, or 7. Cells were stained for bcl-2 (*X axis*) and CD38 (*Y axis*). Only a lack of effect on day 2 is shown for the mutant oligonucleotides.

Fig. 2 Apoptosis assay by flow cytometry with Apo 2.7. WSU-FSCCL cells were injected i.p. and allowed to grow for 5 weeks. Different doses (200, 300, or 400 μg) of AS-c$_\mu$ oligonucleotides or control (*Mut*) oligonucleotides were injected i.p., and ascites were removed at 16 h. Cells from three mice/condition were stained and analyzed for percentage of Apo 2.7 positivity. *Bars*, SD.

mg/kg) of c$_\mu$-AS oligonucleotide or mutated control, as in Fig. 1, and ascites specimens were analyzed. Apoptotic cells were visible in cytospin preparations at 8 h. To quantitate the effect, we used an antibody (Apo 2.7) to a mitochondrial membrane protein that is involved in apoptosis and detected on the surface of early apoptotic cells. Background apoptosis in cells from untreated mice is 1–3%. Increased apoptosis was detected within 4 h after oligonucleotide injection, peaked at 16 h, and was returning to baseline by 24 h (not shown). We therefore examined, at the 16 h peak of apoptosis, the effect of adding increasing amounts of AS oligonucleotides on *in vivo* apoptosis. The data in Fig. 2 demonstrate a dose response with apoptosis maximal at 300 μg.

Down-Regulation of bcl-2 Protein by c$_\mu$ AS Oligonucleotides. We have reported that bcl-2 is down-regulated in WSU-FSCCL cells *in vitro* by c$_\mu$ AS oligonucleotides (18). Our

initial attempts to similarly confirm down-regulation *in vivo* were unsuccessful. Because of the reported bcl-2 half-life of >10 h (5), we had initially examined later time points. By then, however, many cells were already apoptotic. Because the peak of apoptosis is at 16 h, earlier time points were examined. At 8 h, bcl-2 was variably down-regulated in spleen tissue. We postulated that if bcl-2 down-regulation and apoptosis were temporally closely linked, then it would be difficult to detect cells that had low bcl-2 expression but had not undergone apoptosis. Thus, if cells could be trapped in a bcl-2^{low} state by inhibiting apoptosis, then the specific down-regulation of bcl-2 could be demonstrated. Accordingly, mice were inoculated with WSU-FSCCL cells and treated 5 weeks later with 400 μg i.p. of AS or mutated c$_\mu$ oligonucleotide. The caspase inhibitor Z-VAD was administered i.p. 2 h later to block apoptosis. Spleens were removed 8 h after oligonucleotide treatment, and bcl-2 protein levels were assayed relative to human CD38 expression by Western analysis. A representative blot is shown (Fig. 3). In three experiments, each with two animals/experiment, densitometric analysis of the blots revealed that the ratio of bcl-2:CD38 in the presence of Z-VAD was 110.8 ± 22.9 with the control oligonucleotide treatment *versus* 17.8 ± 35.5 for AS c$_\mu$-treated mice.

We used flow cytometric detection of bcl-2 as an alternative method of demonstrating and quantifying bcl-2 down-regulation in human CD45+ cells in spleens from treated and control mice. A sample experiment is shown in Fig. 4, *top panel*. Quantitation confirms the Western blot data that AS c$_\mu$ oligonucleotide treatment of mice leads to reduced bcl-2 protein levels in lymphoma cells in the spleen (Fig. 4, *bottom panel*). This also reveals maximal reduction at the 300-μg dose (*P* < 0.02 for comparison of 300- and 200-μg doses; no significant difference for comparison of 400- and 300-μg doses).

Therapeutic Efficacy of c$_\mu$ AS Oligonucleotides. We then sought to determine the therapeutic efficacy of this immu-

Cμ-AS   Cμ-AS+ZVAD   Cμ-Mut.+ZVAD
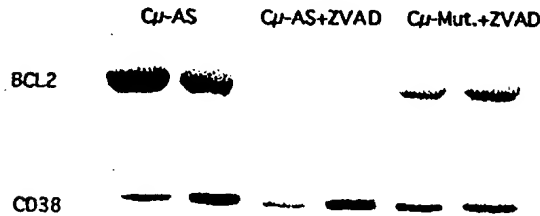
BCL2

CD38

Fig. 3 bcl-2 protein levels in spleen by Western analysis for bcl-2 and CD38. WSU-FSCCL cells were injected i.p. and allowed to grow for 5 weeks. AS-c_μ oligonucleotides or control oligonucleotides (400 μg) were injected i.p., followed 2 h later by i.p. injections of 500 mg of Z-VAD. Spleens were harvested 8 h after oligonucleotide administration for all lanes.
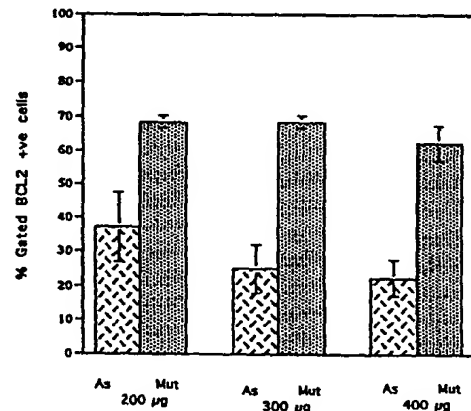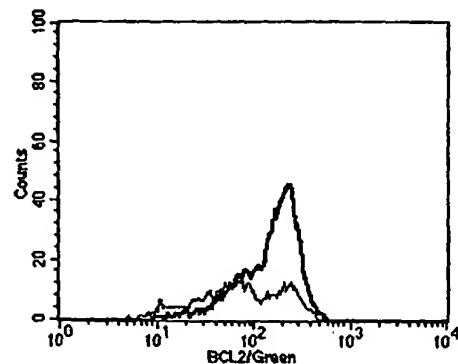

Fig. 4 Flow cytometry for bcl-2 protein expression. Experimental plan was as in Fig. 3, except that doses of oligonucleotides were 200, 300, or 400 μg. Spleens were again harvested 8 h after oligonucleotides (6 h after Z-VAD). Single-cell suspensions were stained for CD45 and bcl-2. *Top panel,* sample flow data in which the *solid line* is control oligonucleotide treated; the *dashed line* is AS-c_μ oligonucleotide treated. *Bottom panel,* quantitation of bcl-2+/CD45+ cells in spleens of three mice/condition. For each dose, AS-c_μ oligonucleotide reduces bcl-2+ cells ($P < 0.01$ versus mutant oligonucleotide). For dose response of AS-c_μ oligonucleotide, $P < 0.012$ for 300 versus 200 μg. Bars, SD.

noglobulin-targeted down-regulation of bcl-2 and induction of apoptosis in WSU-FSCCL cells in *scid* mice. Because cells reappeared in ascites 1 week after an infusion of oligonucleotides at a time when mice had significant tumor burden, we chose to give weekly injections before tumor had visibly developed. Beginning 72 h after cell injection, 200 μg of AS or control oligonucleotides were infused once weekly until mice were dead or euthanized. By 9 weeks, abdominal distension by lymphoma was visible in control, but not c_μ-AS-treated, mice (Fig. 5). As seen in Fig. 6, AS oligonucleotides significantly prolonged survival ($P < 0.001$). Median survival was 10.5 ± 0.5 week for untreated mice and 11.0 ± 0.2 week for mice treated with the control oligonucleotide but extended to 16.7 ± 2.4 weeks for the AS-treated animals. At necropsy, the AS-treated mice generally had similar disease distribution as controls. One mouse, however, was sacrificed with hind limb paralysis found, attributable to meningeal infiltration with lymphoma and had minimal systemic tumor burden, whereas a second was apparently cured, surviving >1 year and remaining PCR negative for bcl-2-c_μ.

A shorter, more dose-intensive schedule of oligonucleotide administration has generally been used. We tested a three times weekly schedule for six doses beginning 1-3 weeks after cell injection (Fig. 7). Median survival was 18.5 weeks if AS was begun by day 8 after cell injection ($P < 0.0001$ versus control), and two of six mice were disease free. If lymphoma was allowed to grow for 3 weeks, efficacy, although still statistically significant ($P = 0.0035$), was markedly diminished to 12.5 weeks median survival, compared with 10.9 weeks in the mutated control oligonucleotide-treated group. Thus, this schedule was more effective if tumor burden was low.

To confirm that these results did not merely reflect local tumor control in ascites, we tested s.c. administration of oligonucleotides *versus* the i.p. route. Oligonucleotides were injected three times weekly for six doses beginning 1 week after cells. In this experiment, median survival for untreated mice was 10.5 weeks, whereas for mutated control oligonucleotides, survival was 10.5 weeks for i.p. and 11.0 weeks for s.c. administration. The AS oligonucleotides prolonged survival to 14.0 weeks with either route of delivery in this experiment. Antisense targeted to c_μ and to the bcl-2 translational start site are equally effective *in vitro,* and we confirmed equal efficacy *in vivo* as well (not

shown). Although the c_μ AS oligonucleotides have four base differences from the corresponding murine IgM sequence, to ensure that no effect on murine IgM was confounding the results, we synthesized the corresponding murine sequence, and this had no therapeutic, nor toxic, effect on the mice (not shown).

DISCUSSION

We have developed a *scid* mouse model for human t(14; 18)-positive follicular lymphoma. The *in vitro* characteristics and immunophenotype of this cell line are largely reproduced *in vivo.* This model resembles the clinical course of low-grade lymphoma in being disseminated to lymph nodes, liver, spleen, and bone marrow, regardless of whether cells are introduced i.p., i.v., or s.c. The median survival of ~10 weeks after injection of 10 million cells is longer than other models of t(14;18)-positive lymphomas (22). This longer time frame is also more
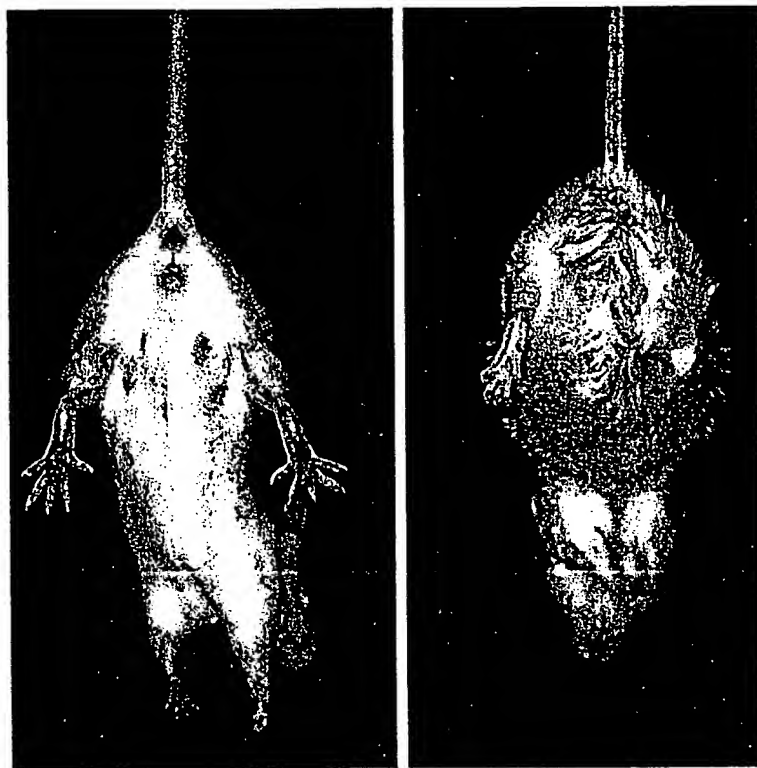
Fig. 5   Mice 9 weeks after inoculation with 1×10^7 WSU-FSCCL cells, treated weekly with 200 μg of oligonucleotides. *Right*, mutated control; *left*, c_μ-AS.

Fig. 6 Survival of oligonucleotide-treated *scid/* FSCCL mice. Mice received injections of 1×10^7 WSU-FSCCL cells i.p. Oligonucleotides were administered i.p. to 10 mice/group, beginning 3 days later at the 200 μg/dose once weekly until death.
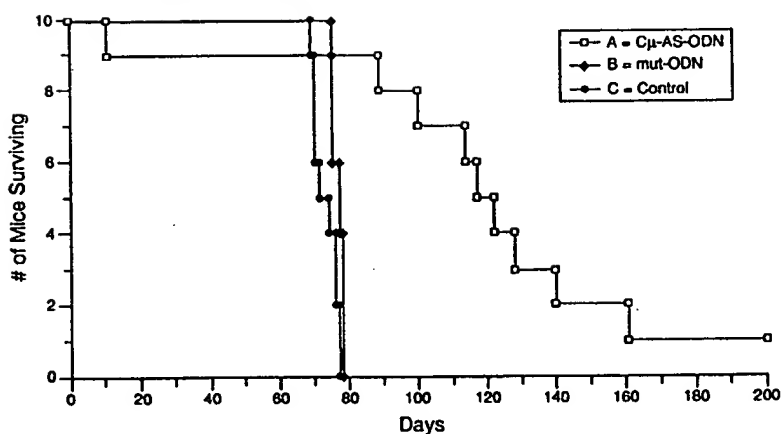

representative of typical lymphoma biology in humans. Meningeal involvement is uncommon in human low-grade non-Hodgkin's lymphoma but occurs in other *scid* leukemia/lymphoma models (23). It also develops in our model, primarily in longer survivors after effective treatment.

Follicular lymphoma is characterized by t(14;18), which leads to dysregulated *bcl-2* gene expression and prolonged lymphoma cell survival (24, 25). bcl-2 is, therefore, a reasonable therapeutic target. AS oligonucleotides targeted to the bcl-2 translational start site have been active in down-regulating bcl-2

and inhibiting cell growth *in vitro* (5–10) and *in vivo* (11–13). bcl-2 is, however, expressed in a variety of normal cells (10, 16). Although short-term down-regulation of bcl-2 has been well tolerated (14), increased apoptosis in these normal tissues may be toxic. In t(14;18) cells, bcl-2 is expressed from a fusion transcript containing the entire bcl-2 coding region and 3' immunoglobulin sequences. Our underlying hypothesis is that AS oligonucleotides targeted to the non-oncogenic immunoglobulin sequences could down-regulate bcl-2 without concerns of short- and long-term toxicity. These oligonucleotides would be specif-

Fig. 7 Survival of oligonucleotide-treated *scid/* FSCCL mice. Mice received injections of 1 × 10^7 WSU-FSCCL cells i.p. Oligonucleotides were administered i.p. to six mice/group, at 200 μg/dose three times/week for six doses, beginning either 1 or 3 weeks later. Mice were then observed for survival without additional treatment.
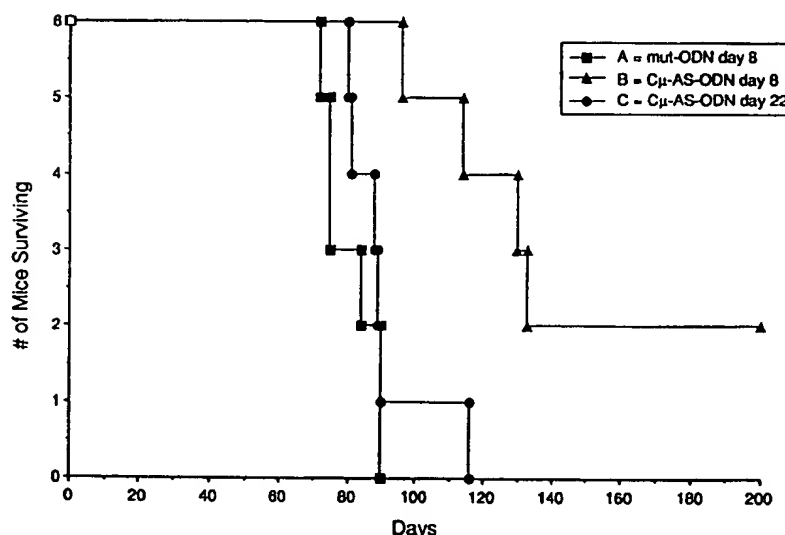
ically toxic to t(14;18)-positive cells, at most leading to a transient decrease in immunoglobulin expression by B cells. Such hypogammaglobulinemia would not be expected to be clinically significant.

In WSU-FSCCL cells, the predominant RNA transcript contains bcl-2 fused to J_{11} and then c_μ. We reported previously that AS oligonucleotides targeted to the C_{11}-2 region of c_μ effectively down-regulated bcl-2 and induced apoptosis in FSCCL cells *in vitro* (18). Here we show that these AS oligonucleotides targeted 3' of the bcl-2 coding region prolong the survival of *scid*-FSCCL mice. Although it may not be surprising that i.p. injection of AS oligonucleotides is able to clear ascites of human cells, we have also demonstrated systemic effects of i.p. injection by measuring effects in the spleen. Further, s.c. oligonucleotide injection gave similar survival results as i.p. injection.

As expected, apoptosis is induced by down-regulation of bcl-2. The time course of apoptosis is more rapid than expected from the previously reported $t_{1/2}$ of bcl-2 protein (5). This suggests that the bcl-2 half-life may be shorter in these cells, although this has not been formally determined. By blocking the execution of caspase-mediated apoptosis with the caspase inhibitor Z-VAD, we have prevented this rapid cell death and trapped cells in a bcl-2^{low} but viable state.

Although these AS oligonucleotides are active *in vivo*, they rarely cure the mice at this dose and schedule. Higher doses, altered schedules of administration, and/or prolonged therapy may be beneficial. The AS oligonucleotides are less effective with higher tumor burden, which suggests that attaining minimal residual disease with chemotherapy might then permit more efficacious use of these oligonucleotides. In addition, because bcl-2 prevents apoptosis, including that induced by chemotherapy, bcl-2 down-regulation has been shown to be chemosensitizing (7, 9, 11, 26). Thus, combining AS oligonucleotides to down-regulate bcl-2 along with chemotherapy is a rational approach we are investigating to enhance AS effects.

REFERENCES

1. Weiss, L. M., Warnke, R. A., Sklar, J., and Cleary, M. D. Molecular analysis of the t(14;18) chromosomal translocation in malignant lymphoma. N. Engl. J. Med., 317: 1185–1189, 1987.

2. Ngan, B-Y., Nourse, J., and Cleary, M. D. Detection of chromosomal translocation t(14;18) within the minor cluster region of BCL-2 by polymerase chain reaction. Blood, 73: 1759–1762, 1989.

3. Graninger, W. B., Seto, M., Boutain, B., Goldman, P., and Korsmeyer, S. J. Expression of BCL-2 and BCL-2-Ig fusion transcripts in normal and neoplastic cells. J. Clin. Investig., 80: 1512–1515, 1987.

4. Seto, M., Jaeger, U., Hockett, R. D., Graninger, W., Bennett, S., Goldman, P., and Korsmeyer, S. J. Alternative promoters and exons, somatic mutation and deregulation of the BCL-2-Ig fusion gene in lymphoma. EMBO J., 7: 123–131, 1988.

5. Reed, J. C., Stein, C., Subasinghe, C., Haldar, S., Croce, C. M., Yum, S., and Cohen, J. Antisense-mediated inhibition of BCL2 proto-oncogene expression and leukemic cell growth and survival: comparisons of phosphodiester and phosphorothioate oligodeoxynucleotides. Cancer Res., 50: 6565–6570, 1990.

6. Smith, M. R., Abubakr, Y., Mohammad, R., Xie, T., Hamdan, M., and Al-Katib, A. Antisense oligodeoxyribonucleotide down-regulation of bcl-2 gene expression inhibits growth of the low-grade non-Hodgkin's lymphoma cell line WSU-FSCCL. Cancer Gene Ther., 2: 207–212, 1995.

7. Keith, F. J., Bradbury, D. A., Zhu, Y-M., and Russell, N. H. Inhibition of bcl-2 with antisense oligonucleotides induces apoptosis and increases sensitivity of AML blasts to ara-C. Leukemia (Baltimore), 9: 131–138, 1995.

8. Tormo, M., Tari, A. M., McDonnell, T. J., Cabanillas, F., Garcia-Conde, J., and Lopez-Berestein, G. Apoptotic induction in transformed follicular lymphoma cells by Bcl-2 down-regulation. Leuk. Lymphoma, 30: 367–379, 1998.

9. Zangemeister-Wittke, U., Schenker. T., Luedke, G. H., and Stahel, R. A. Synergistic cytotoxicity of bcl-2 antisense oligodeoxynucleotides

and etoposide, doxorubicin and cisplatin on small-cell lung cancer cell lines. Br. J. Cancer, *78:* 1035–1042, 1998.

10. Campos, L., Sabido, O., Rouault, J-P., and Guyotat, D. Effects of BCL-2 antisense oligodeoxynucleotides on *in vitro* proliferation and survival of normal marrow progenitors and leukemic cells. Blood, *84:* 595–600, 1994.

11. Jansen, B., Schlagbauer-Wadl, N., Brown, B. D., Bryan, R. N., van Elsas, A., Muller, M., Wolff, K., Eichler, H. G., and Pehamberger, H. BCL-2 antisense therapy chemosensitizes human melanoma in scid mice. Nat. Med., *4:* 232–234, 1998.

12. Miyake, H., Tolcher, A., and Gleave, M. E. Antisense bcl-2 oligooxynucleotides inhibit progression to androgen independence after castration in the Shionogi tumor model. Cancer Res., *59:* 4030–4034, 1999.

13. Gleave, M., Tolcher, A., Miyake, H., Nelson, C., Brown, B., Beraldi, E., and Goldie, J. Progression to androgen independence is delayed by adjuvant treatment with antisense Bcl-2 oligodeoxynucleotides after castration in the LNCaP prostate tumor model. Clin. Cancer Res., *5:* 2891–2898, 1999.

14. Webb, A., Cunningham, D., Cotter, F., Clarke, P. A., diStefano, F., Ross, P., Corbo, M., and Dziewanowski, Z. BCL-2 antisense therapy in patients with non-Hodgkin's lymphoma. Lancet, *349:* 1137–1141, 1997.

15. Kamada, S., Shimono, A., Shinto, Y., Tsujimura, T., Takahashi, T., Noda, T., Kitamura, Y., Kondoh, H., and Tsujimoto, Y. bcl-2 deficiency in mice leads to pleiotropic abnormalities: accelerated lymphoid cell death in thymus, in spleen, polycystic kidney, hair hypopigmentation, and distorted small intestine. Cancer Res., *55:* 354–359, 1995.

16. Bonati, A., Albertini, R., Garau, D., Pinelli, S., Lunghi, P., Almici, C., Carlo-Stella, C., Rizzoli, V., and Dall-Aglio, P. *BCL2* oncogene protein expression in human hematopoietic precursors during fetal life. Exp. Hematol., *24:* 459–465, 1996.

17. Nakayama, K., Nakayama, K-I., Negishi, I., Kuida, K., Sawa, H., and Loh, D. Y. Targeted disruption of Bcl-2β in mice: occurrence of gray hair, polycystic kidney disease, and lymphocytopenia. Proc. Natl. Acad. Sci. USA, *91:* 3700–3704, 1994.

18. Smith, M. R., Xie, T., Zhou, Z., and Joshi, I. Antisense oligonucleotides complementary to immunoglobulin sequences of BCL2/Ig fusion transcript induce apoptosis of t(14;18) lymphoma cells. Br. J. Haematol., in press, 2001.

19. Cleary, M. L., Smith, S. D., and Sklar, J. Cloning and structural analysis of DNAs for bcl-2 and a hybrid bcl-2/immunoglobulin transcript resulting from the t(14;18) translocation. Cell, *47:* 19–28, 1986.

20. Dorai, H., and Gillies, S. D. The complete nucleotide sequence of a human immunoglobulin genomic *Cu* gene. Nucleic Acids Res., *17:* 6412, 1989.

21. Kawakami, T., Takahashi, N., and Honjo, T. Complete nucleotide sequence of mouse immunoglobulin *mu* gene and comparison with other immunoglobulin heavy chain genes. Nucleic Acids Res., *8:* 3933–3945, 1980.

22. Cotter, F. E., Johnson, P., Hall, P., Pocock, C., Mahdi, N. A., Cowell, J. K., and Morgan, G. Antisense oligonucleotides suppress B-cell lymphoma growth in a SCID-mouse model. Oncogene, *9:* 3049–3055, 1994.

23. Ratajczak, M. Z., Kant, J. A., Luger, S. M., Hijaya, N., Zhang, J., Zon, G., and Gewirtz, A. M. *In vivo* treatment of human leukemia in a scid mouse model with c-myb antisense oligodeoxynucleotides. Proc. Natl. Acad. Sci. USA, *89:* 11823–11827, 1992.

24. McDonnell, T. J., Deane, N., Platt, F. M., Nunez, G., Jaeger, U., McKearn, J. P., and Korsmeyer, S. J. BCL-2-immunoglobulin transgenic mice demonstrate extended B cell survival and follicular lymphoproliferation. Cell, *57:* 79–88, 1989.

25. Strasser, A., Harris, A. W., and Cory, S. Eμ-*bcl*-2 transgene facilitates spontaneous transformation of early pre-B and immunoglobulin-secreting cells but not T-cells. Oncogene, *8:* 1–9, 1993.

26. Kitada, S., Takayama, S., deRiel, K., Tanaka, S., and Reed, J. C. Reversal of chemoresistance of lymphoma cells by antisense-mediated reduction of *bcl-2* gene expression. Antisense Res. Dev., *4:* 71–79, 1994.

Tumor Growth Inhibition in Vivo and G_2/M Cell Cycle Arrest Induced by Antisense Oligodeoxynucleotide Targeting Thymidylate Synthase

RANDAL W. BERG, MELANIE WERNER, PETER J. FERGUSON, CARL POSTENKA, MARK VINCENT, D. JAMES KOROPATNICK, and ELKE BEHREND

Cancer Research Laboratories, The London Regional Cancer Centre, London, Ontario, Canada (R.W.B., M.W., P.J.F., C.P., E.B., M.V., D.J.K.); and Departments of Oncology (M.V., D.J.K.), Microbiology and Immunology, Pathology, and Pharmacology and Toxicology (D.J.K.), The University of Western Ontario, London, Ontario, Canada

ABSTRACT

Chemotherapeutic agents targeting thymidylate synthase (TS) are effective against human tumors. Efficacy is limited by drug resistance, often mediated by TS overexpression. Treatment of HeLa cells in vitro with an antisense oligodeoxynucleotide (ODN 83) targeting human TS mRNA reduces TS mRNA and protein levels, inhibits cell proliferation, and sensitizes cells to TS-targeting drugs (Ferguson et al., 1999). The present study investigates the mechanism by which ODN 83 inhibits cell proliferation and examines its antitumor efficacy in vivo. ODN 83 treatment did not induce apoptosis in HeLa cells in vitro but caused accumulation of cells at G_2/M. In contrast, TS-targeting chemotherapeutics arrest at G_1 or S. Antisense down-regulation reduced TS mRNA levels in human colon cancer (HT29) cells by 40% in vitro, resulted in G_2/M arrest, and reduced proliferation without enhanced cell death. Growth of HT29 tumors in immunocompromised mice was significantly inhibited when antisense ODN 83 treatment began promptly after tumor implantation and was accompanied by a 40% reduction in TS protein levels. Growth of tumors allowed to reach 400 mm³ prior to ODN administration was unaffected by antisense ODN 83. Radiolabeled ODNs were localized to the tumor periphery but evenly distributed in normal tissue. Thus, down-regulation of TS mRNA and protein by antisense ODN treatment exerts a novel G_2/M cell cycle block without increasing cell death and inhibits HT29 tumor cell growth in vivo. Antisense ODN 83 may be an effective therapy for colon carcinoma, alone or in combination with TS-targeting cytotoxic drugs.

Thymidylate synthase (TS) is an essential enzyme for de novo synthesis of thymidylate and is required for DNA replication. It plays an important role in cell proliferation and is an important target for antitumor therapies designed to reduce the proliferative capacity of cancer cells (Danenberg et al., 1999). Chemotherapeutic drugs that target TS include 5-fluorouracil (5-FU) and raltitrexed (Tomudex) (Papamichael, 1999). Although these drugs have had clinical success, resistance to TS inhibitors often develops both in vitro and in vivo (Gorlick and Bertino, 1999). Drug resistance can be mediated by a variety of mechanisms, including increased TS levels resulting from increased TS transcription (Shibata et al., 1998) and translation (Keyomarsi et al., 1993), and is a major obstacle to clinical efficacy.

A growing number of cellular components are being targeted for therapeutic down-regulation by use of antisense oligodeoxynucleotides (ODNs). Antisense ODNs specifically decrease protein expression by base-pairing with a specific mRNA to block translation and/or induce cleavage by ribonuclease H (Stein and Cheng, 1993). Antitumor effects have been observed in tumor-bearing animals in response to antisense ODNs against targets as varied as the proto-oncogenes c-myb (Del Bufalo et al., 1996) and c-myc (Leonetti et al., 1996), matrilysin (matrix metalloproteinase-7) (Hasegawa et al., 1998), the antiapoptotic protein Bcl-2 (Miayake et al., 2000), and protein kinase Cα (Dean et al., 1996). Phosphorothioate ODNs administered to mice were found at highest concentrations in the liver and kidney and were stable in most tissues for more than 48 h (Agrawal et al., 1991; Saijo et al., 1994). Although the accumulation and stability of ODNs in tumor tissue in vivo were demonstrated in these two studies, their distribution within a tumor mass has not been described.

We have previously shown that treatment of HeLa cells in

ABBREVIATIONS: TS, thymidylate synthase; ODN, oligodeoxynucleotide; 5-FU, 5-fluorouracil; 5-FUdR, 5-fluorodeoxyuridine; 5-FdUMP, 5-fluorodeoxyuridine monophosphate; RT-PCR, reverse transcriptase-polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

vitro with an antisense ODN targeting TS decreases TS mRNA and protein lev ls, inhibits cell proliferation, and sensitizes HeLa cells to 5-FU, 5-fluorodeoxyuridine (5-FUdR), and raltitrexed (Ferguson et al., 1999). Antisense ODNs against TS might be expected to block DNA synthesis and arrest cells in G_1 or early S phase, similar to the action f 5-FU (Inaba and Mitsuhashi, 1994) and raltitrexed (Matsui et al., 1996; Yin et al., 1999). However, TS protein binds to a variety of mRNA molecules, including those encoding p53 (Chu et al., 1996; Ju et al., 1999), c-myc (Chu et al., 1995), and TS itself (Chu et al., 1991, 1994). This has raised the possibility that post-transcriptional regulation of mRNA metabolism by TS protein might control not only TS protein production but also that of cell cycle regulatory proteins. Treatment of cells with TS antisense ODNs to decrease both TS mRNA and protein levels may lead to cell cycle perturbations that are not predicted from experiments using protein-targeting drugs that act after mRNA translation.

To examine the cytotoxic and cytostatic effects of targeting TS with antisense ODNs, we have measured apoptosis in H La cells and analyzed the cell cycle distribution of HeLa and HT29 carcinoma cells treated in vitro with TS antisense ODN 83. In addition, to test the hypothesis that antisense down-regulation of TS would be an effective antitumor strategy in vivo, we have assessed the antitumor activity of TS antisense ODN 83 on the growth of HT29 tumor explants in immunocompromised mice. We report that the TS antisense ODN 83 has novel and potentially therapeutically exploitable effects on human tumor cell cycle and viability in vitro and in vivo.

Materials and Methods

Cell Culture. HeLa and HT29 cells (from the American Type Culture Collection, Manassas, VA) were grown in Dulbecco's modified Eagle's medium and RPMI1640 (respectively) containing 10% fetal bovine serum at 37°C in a humidified atmosphere of 5% CO_2. All tissue culture reagents were from Invitrogen Canada (Burlington, ON, Canada).

Oligodeoxynucleotides and Transfection. The TS antisense ODN 83 (5'-GCCAGTGGCAACATCCTTAA-3') is complementary to the sequence 136 to 155 base pairs downstream of the translation stop site in the 3' untranslated region of human TS mRNA. There are no other known mRNAs (including mouse TS) with sequences complementary to ODN 83. The control scrambled ODN 32 (5'-ATGCGCCAACGGTTCCTAAA-3') has the same base composition in random order. For in vivo studies, ODNs with phosphorothioate linkages between the six nucleotides at both the 5'- and 3'-ends were purchased from BioCorp Inc. (Montreal, QC, Canada). For in vitro studies, fully phosphorothioated ODNs with 2'-methoxy-ethoxy modification on the six nucleotides at both the 5'- and 3'-ends were generously provided by Dr. N. Dean (ISIS Pharmaceuticals, Carlsbad, CA).

HeLa and HT29 cells were transfected with ODNs using LipofectAMINE (Invitrogen Canada), as described (Ferguson et al., 1999), or with LipofectAMINE 2000 (Invitrogen Canada) as described below. For HT29 cells, preliminary experiments indicated that LipofectAMINE 2000 was the superior lipid formulation and was effective at 1 to 5 μg/ml, although nonspecific toxicity was apparent at the higher doses. Concentrations of ODN 83 from 50 to 200 nM were found to be effective at inhibiting HT29 cell proliferation, with improved activity at the higher concentrations. For proliferation assays, HT29 cells were plated at 2×10^5 cells per 25-cm^2 flask in 2 ml of medium. On the following day, a 6× transfection mix was prepared containing 600 nM ODN and 6 μg/ml LipofectAMINE

2000 in serum-free medium. After incubation for 15 min at room temperature, 5 volumes of medium with 10% serum were added, and the medium on the cells was replaced with the 1× transfection mix. For flow cytometry samples and RNA preparation, cells were plated at 1 to 2×10^6 cells per 75-cm^2 flask in 5 ml of medium. The next day, 1 ml of a DNA/lipid mixture in serum-free medium was added directly to each 75-cm^2 flask to yield final concentrations of 200 nM ODN and 2 μg/ml lipid.

Animal Studies. Female nude mice (N:NIH-bg-nu-xid), purchased from Charles River Laboratories (St. Constant, QC, Canada), were housed and cared for according to standards of the Canadian Council for Animal Care and were used under a protocol approved by the University of Western Ontario Council on Animal Care. TS-inhibitory cytotoxic drugs are a mainstay in the treatment of colon cancer (Papamichael, 1999), and preliminary studies indicated that human HT29 colon carcinoma cells grow well in these immunocompromised mice (E. Behrend, unpublished observations). To assess the effect of ODN on tumor growth, 4- to 6-week-old mice were injected subcutaneously in the right flank with 5×10^6 HT29 cells on day 0. Every 2nd day beginning on day 1, mice were injected intraperitoneally with ODNs dissolved in 150 mM NaCl. Tumors were measured in two perpendicular dimensions with a caliper every 4 days, and tumor volume was calculated using the formula volume = length × width^2 × π/6. To measure their effect on larger, established tumors, ODNs were administered to mice when the tumors achieved a size of 400 mm^3.

Oligodeoxynucleotide Labeling and Distribution Analysis. ODNs (2 μg) were end-labeled to a specific activity of 2×10^8 cpm/μg with [γ-^{32}P]adenosine triphosphate (specific activity, 7000 Ci/mmol, ICN Pharmaceuticals, Inc., Costa Mesa, CA) using 20 units of T4 polynucleotide kinase (New England Biolabs, Beverly, MA) for 10 min at 37°C. Unincorporated radionucleotide was removed using a Sephadex G50 NICK column (Amersham Pharmacia Biotech AB, Uppsala, Sweden). Labeled ODN (200 ng) was mixed with 0.5 mg of unlabeled ODN for each injection. Mice that had been treated with ODN every 2nd day beginning the day after tumor cell implantation, and who were bearing HT29 tumors approximately 800 mm^3 in volume, were injected intraperitoneally two times 4 days apart with the labeled ODN and then sacrificed 2 days later. Various tissues were fixed in neutral-buffered formalin and embedded in paraffin, and 5-μm sections were stained with hematoxylin and eosin. PhosphorImage screens were exposed to tissue sections for 3 days and radioactive decay images captured on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Thymidylate Synthase Quantitation. A [6-^{3}H]5-FdUMP binding assay (Spears and Gustavsson, 1988) was used to quantitate TS in tumor samples, as described (Ferguson et al., 1999). Tumors were obtained from mice treated with ODN every second day for 28 days, beginning on day 1 after tumor cell implantation. Cell lysates from frozen tumors were prepared by homogenization in 100 mM potassium phosphate, pH 7.4, followed by one cycle of freezing, thawing, and sonication. Supernatants were obtained following centrifugation at 6500g for 30 min at 4°C. Protein concentrations were determined using a protein assay kit (Bio-Rad, Hercules, CA). Aliquots containing 100 μg of total protein were incubated with 75 μM methylene tetrahydrofolate, 100 mM 2-mercaptoethanol, and 15 nM [6-^{3}H]5-FdUMP (specific activity, 18.6 Ci/mmol, Moravek Biochemicals, Brea, CA), in 50 mM potassium phosphate (pH 7.4) for 30 min at 37°C, and the reaction was stopped with 1 ml of albumin-coated, acidified charcoal for 10 min at room temperature. The slurry was centrifuged two times at 5000g for 30 min at 22°C to remove particulate matter, and 400-μl aliquots of the cleared supernatant were assayed by scintillation counting. [6-^{3}H]5-FdUMP bound to TS, and therefore unavailable for precipitation with charcoal, was quantitated.

Apoptosis Assay. HeLa cells were treated with 4 μg/ml LipofectAMINE and 50 nM ODN for 6 h, trypsinized, and plated onto coverslips in 12-well plates at 1×10^4 cells/well in culture medium.

Because ODN 83 effectively suppresses, within 24 h, the normal increase in HeLa cell number resulting from proliferation (Ferguson et al., 1999), an earlier time-point was chosen to assess apoptosis. After 15 h, cells were air dried and fixed in 4% paraformaldehyde for 30 min, washed with phosphate-buffered saline, permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 2 min at 4°C, and washed with phosphate-buffered saline. Apoptotic cells were detected using the In Situ Cell Death detection kit (Roche Diagnostics, Laval, QC, Canada) and Fast Red (Sigma, St. Louis, MO) as the chromogenic substrate. More than 500 cells were scored as either apoptotic (stained red) or nonapoptotic for each condition.

Flow Cytometry. Cells were collected at 24, 36, 48, and 72 h after ODN treatment, washed with phosphate-buffered saline, fixed in 75% ethanol for 15 min at room temperature, and washed again. Cells treated with 1 μM raltitrexed or 0.1 mM 5-FU for 2 to 4 h, then washed and cultured for 2 days in drug-free medium, were similarly collected. The cells were stained with propidium iodide [0.02 mg/ml in phosphate-buffered saline with 0.1% (v/v) Triton X-100 and 0.2 mg/ml deoxyribonuclease-free ribonuclease A] and analyzed on a Beckman Coulter XL-MCL flow cytometer. At least 10,000 single cells were analyzed for each condition. Analysis regions were set manually to determine the proportion of cells in G$_0$/G$_1$, S, and G$_2$/M cell cycle phases. Alternatively, MultiCycle (version 3.0) software (Phoenix Flow Systems, San Diego, CA) was used to analyze the cell cycle distribution.

Quantitation of TS mRNA by RT-PCR. Total RNA was isolated from HT29 cells treated with ODNs using TRIzol (Invitrogen Canada), and 2 μg of RNA reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Invitrogen Canada). Two percent of the cDNA produced was used as template for PCR with the primers GAP-for (5'-TATTGGGCGCCTGGTCACCA-3') and GAP-rev (5'-CCACCTTCTTGATGTCATCA-3') for GAPDH, or TS-for (5'-TTTTGGAGGAGTTGCTGTGG-3') and TS-rev (5'-TGTGCATCTC-CCAAAGTGTG-3') for TS. PCR cycling parameters were 3 min at 94°C, followed by 23 cycles of 30 s at 94°C, 30 s at 58°C, 45 s at 72°C, and a 7-min 72°C extension. Products were resolved on 1.5% agarose gels and stained with ethidium bromide. Quantitation of images captured using the ImageMASTER VDS gel documentation system (Amersham Pharmacia Biotech) was done with ImageQuant version 5.1 (Molecular Dynamics).

Statistical Analysis. Statistical significance within experiments was determined using Student's t test ($p < 0.05$). All experiments were repeated at least twice.

Results

Apoptosis of HeLa Cells is Unaffected by Oligodeoxynucleotide Treatment. In order to examine more closely the mechanism by which treatment with ODN 83 inhibits cell proliferation, the terminal deoxynucleotidyl transferase dUTP nick-end-labeling assay was used to determine whether apoptosis was induced in HeLa cells. Cisplatin treatment was included as a positive control and induced apoptosis in 85% of exposed cells after a 15 h treatment (Table 1). The low levels of apoptosis in HeLa cells were unchanged at 15 h after treatment with ODN 83, suggesting that the antiproliferative effects of the antisense ODN 83 might be due to a cytostatic, rather than an apoptotic, mechanism. Apoptosis levels were also not increased at 24 or 39 h after ODN 83 treatment (data not shown).

TS Antisense Oligodeoxynucleotide Induces a G$_2$/M Cell Cycle Block in HeLa Cells. Flow cytometric analysis was used to examine the cell cycle distribution of HeLa cells treated for various times with ODNs. There were substantial increases in the fraction of cells in G$_2$/M at 24, 36, and 48 h after treatment with the TS antisense ODN 83, compared

with the control scrambled ODN 32 (25–46 versus 18–22%, respectively) (Fig. 1). Increases in the fraction of cells in S phase at 24 and 36 h (19 and 12% in ODN 32-treated cells compared with 35% in ODN 83-treated cells) were essentially reversed by 48 h. Accumulation of cells in G$_2$/M was seen as early as 24 h, whereas the profile at 72 h closely resembled that of control cells. In contrast, treatment of HeLa cells with raltitrexed (1 μM for 2 h, followed by culture in drug-free medium for 48 h) resulted in cell cycle arrest exclusively in early S phase: 76% of raltitrexed-treated cells versus 22% of control cells were in S phase, whereas 23% of raltitrexed-treated cells were in G$_0$/G$_1$, versus 61% of control cells.

Effects of TS Antisense ODN 83 on HT29 Cells In Vitro. To determine whether the observed G$_2$/M arrest was p53-dependent, the effects of ODN 83 on a p53 mutant cell line, the HT29 human colon carcinoma, were examined. RT-PCR analysis showed that treatment of HT29 cells with the TS antisense ODN 83 reduced the levels of cytoplasmic TS mRNA but not GAPDH mRNA (Fig. 2A). Quantitation of the TS PCR product normalized to the GAPDH product from two independent RT-PCR experiments revealed 37% (±1.53) and 43% (±4.78) reductions in TS mRNA at 24 and 48 h, respectively, in cells treated with the TS antisense ODN 83 compared with cells treated with the control scrambled ODN 32. Flow cytometric analysis showed that HT29 cells, similar to HeLa cells, were blocked in G$_2$/M at 48 h after treatment with TS antisense ODN 83 (Fig. 2C). Quantitation of these data indicated that 43.0% of the ODN 83-treated cells were in the G$_0$/G$_1$, 31.2% in S, and 25.8% in G$_2$/M phase, compared with 62.3, 25.2, and 12.5%, respectively, for ODN 32-treated cells. Again similar to HeLa cells, raltitrexed and 5-FU both induced G$_1$/S phase arrest in HT29 cells (Fig. 2, D and E). Treatment with TS antisense ODN 83 (100 nM) for 4 days inhibited proliferation of HT29 cells by 50.2% (S.E.M. = 4.75, $n = 4$), compared with cells treated with the control scrambled ODN 32. Thus, antisense ODN-mediated down-regulation of TS mRNA and protein in HeLa and HT29 cells activates a novel G$_2$/M cell cycle block and inhibits cell proliferation in vitro.

Effect of TS Antisense ODN 83 on HT29 Tumor Growth In Vivo. Growth of human HT29 colon carcinoma cells in immunocompromised mice was examined to determine the antitumor efficacy of TS antisense as a single agent therapy. Compared with mice treated with the control scram-
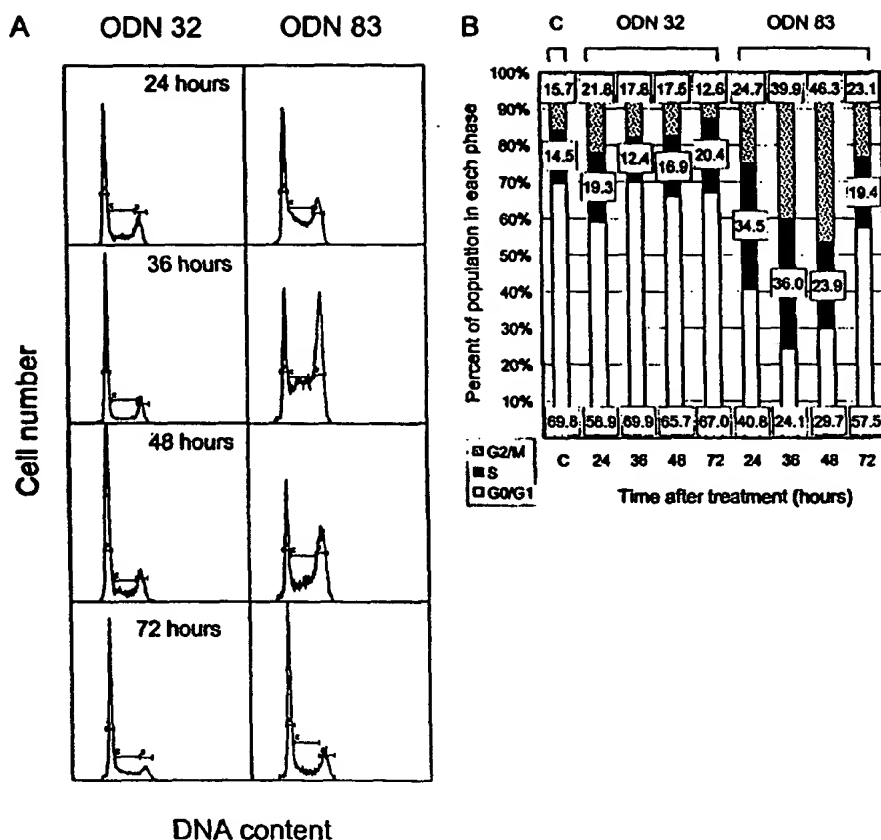
Fig. 1. Flow cytometric analysis of cell cycle distribution of ODN-treated HeLa cells. HeLa cells were collected at the indicated times after treatment with control scrambled ODN 32 (A, left) or TS antisense ODN 83 (A, right), stained with propidium iodide, and analyzed by flow cytometry. B, the percentage of cells in the G_0/G_1 (open region), S (filled region), and G_2/M (dotted region) cell cycle phases for control untreated HeLa cells (C), and for the histograms shown in A.

bled ODN 32, treatment with the TS antisense ODN 83 significantly inhibited tumor growth over the course of 4 weeks (Fig. 3). Growth of tumors in mice treated with the control scrambled ODN 32 was not significantly different from those injected with saline ($p = 0.217$ to $p = 0.982$ for differences in tumor volume on days 7–27, Student's t test, $n = 5$). Tumor growth delay was caused by the antisense ODN 83 at 7.5 or 11.25 mg/kg of body weight when the ODN was administered every 2 days commencing the day after tumor implantation. However, there was no effect at either concentration of ODN against tumors that had been allowed to grow untreated to a size of 400 mm³ prior to commencing treatment (Fig. 4). The ODNs were well tolerated, without significant delays in normal weight gain ($p = 0.37$ to $p = 0.76$ for differences in mean body weight of mice treated for 28 days with saline, antisense ODN 83, or control ODN 32, Student's t test, $n = 10$).

Distribution of Oligodeoxynucleotides in Vivo. We hypothesized that ODN delivery or penetration into larger tumors might be a limiting factor in this therapy. To examine this question, end-labeled ODN was used as a tracer for injection into mice bearing HT29 tumors. The radiolabel was evenly distributed in normal spleen, kidney, and liver, but it was relatively concentrated around the periphery of the tumor (Fig. 5A). There was a higher degree of cellularity around the periphery f the tumors (Fig. 5C) compared with the interiors (Fig. 5D), which were necrotic, as evidenced by cell shrinkage, increased extracellular space, and decreased hematoxylin-staining nuclei. The morphology of tumors from mice treated with the control and the antisense ODNs was

similar. There was no significant difference in the mitotic index between tumors in the two groups of animals.

Decreased TS Levels in HT29 Tumors in Nude Mice Treated with Antisense Oligodeoxynucleotide. To test whether in vivo administration of TS antisense ODN 83 down-regulates TS expression in HT29 tumors, a [6-³H]5-FdUMP binding assay was used to measure TS levels. Tumors were dissected from mice that had been treated with ODN every 2nd day for 28 days (the experiment shown in Fig. 3B) and the levels of TS were measured as described under *Materials and Methods*. Systemic administration f TS antisense ODN 83 caused a 43% decrease in TS protein levels within the tumors, compared with tumors from mice treated with the control scrambled ODN 32 (Table 2).

Discussion

We report that use of an antisense strategy to deplete human tumor cells of TS mRNA, rather than inhibiting the activity of existing TS enzyme with cytotoxic drugs, induced a distinctive response in cultured tumor cell lines. Antisense ODN treatment blocked cells at G_2/M without appreciable arrest at G_1/S and suppressed proliferation in the absence of a measurable increase in apoptosis. The reduced fraction of cells in G_0/G_1 at an early time following antisense ODN treatment (24 h) and the progressive increase in the fraction in G_2/M up to 48 h indicate that blockage at G_1/S followed by synchronous progression through the cell cycle cannot be invoked as a explanation. Between 48 and 72 h after antisense ODN 83 treatment, the proportion of cells in each cell
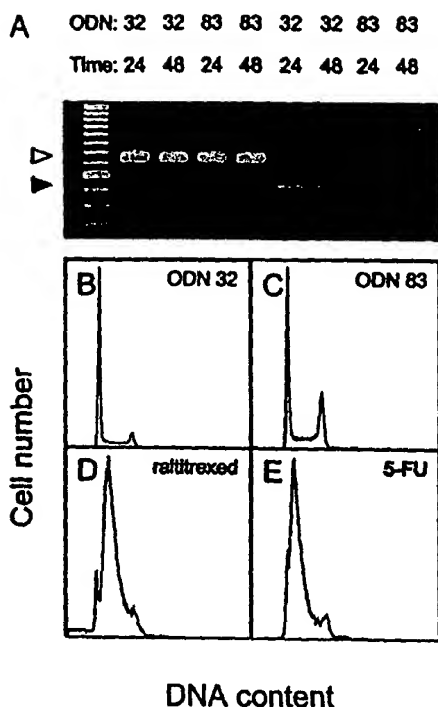
Fig. 2. TS mRNA levels and cell cycle profile in HT29 cells treated with ODNs in vitro. A, RT-PCR analysis of GAPDH (open arrowhead) and TS (filled arrowhead) mRNA levels in HT29 cells 24 and 48 h after treatment with control scrambled ODN 32 or TS antisense ODN 83. B–E, HT29 cells treated for 2 days with 200 nM control scrambled ODN 32 (C) or TS antisense ODN 83 (D), or HT29 cells treated for 4 h with 1 µM raltitrexed (E) or 0.1 mM 5-FU (F), then cultured for 2 days in drug-free medium. Cells were stained with propidium iodide and analyzed by flow cytometry.
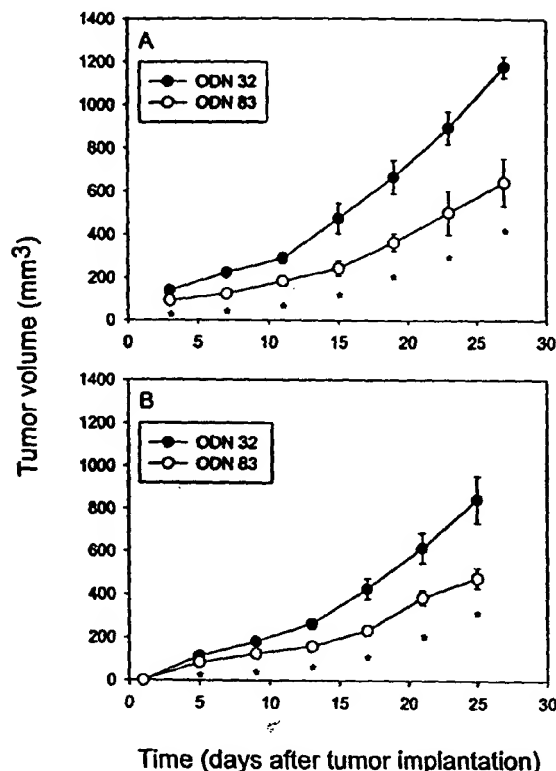
Fig. 3. Antitumor effect of systemic administration of TS antisense ODN 83. Nude mice were injected subcutaneously with HT29 tumor cells on day 0. On every 2nd day beginning on day 1, mice were injected intraperitoneally with 7.5 (A) or 11.25 (B) mg/kg of body weight of control scrambled ODN 32 (filled circles) or TS antisense ODN 83 (open circles), and tumors were measured every 4 days. Shown are the average tumor volume (± standard error) from 15 (A) or 5 (B) mice. *Significant differences ($p < 0.05$) from mice treated with the control scrambled ODN 32.

cycle phase returned to control levels. We previously reported that the proliferation rate of antisense ODN 83-treated cells returned to normal at that time (Ferguson et al., 1999). Thus, the antisense ODN-mediated decrease in TS mRNA and protein corresponds directly with G₂/M arrest and reduced proliferation.

Several molecules are known to play critical roles in mediating arrest at the G₂/M boundary, particularly p53 (Agarwal et al., 1995; Stewart et al., 1995) and its downstream effector p21waf1 (El Deiry et al., 1993). The two human cell lines studied here, HeLa (cervical carcinoma) and HT29 (colon carcinoma), express widely disparate p53 levels. p53 is exceptionally low in HeLa cells, possibly related to unusual instability of the protein due to papillomavirus E6 protein expression (Hamada et al., 1996). Like many other human tumor cell lines, HT29 cells express high levels of mutant p53 (Rodrigues et al., 1990). The observation of a G₂/M block induced by antisense ODN treatment in cells with both high and low p53 expression suggests a p53-independent mechanism, reminiscent of the lack of dependence on p53 of thymineless death induced by direct inhibitors of TS protein (Munoz-Pinedo et al., 2001). The accumulation of cells in G₂/M, as opposed to G₁/S arrest induced by raltitrexed and 5-FU (Fig. 2) (Inaba and Mitsuhashi, 1994; Matsui et al., 1996), suggests that TS antisense treatment has consequences other than simply limiting the supply of thymidylate for DNA synthesis. Direct inhibitors of TS reduce enzyme activity by inactivating pre-existing TS protein, without di-

rectly influencing TS mRNA levels, and often result in increased TS mRNA translation. Antisense targeting, on the other hand, decreases both protein and mRNA levels. The association between decreased TS mRNA and protein levels and G₂/M cell cycle arrest observed in response to antisense targeting suggests that TS mRNA, TS protein, or both, mediate functions additional to catalysis of thymidylate production.

The complexity of control of TS expression provides multiple points where antisense treatment could interfere with additional functions to influence cell cycle and apoptotic pathways in novel ways. For example, TS mRNA levels and enzyme activity increase 10- to 20-fold as cells progress through cell cycle (Navalgund et al., 1980), while TS gene transcription rate is up-regulated only two to four times, suggesting that multiple post-transcriptional mechanisms play a major role in TS regulation (Johnson, 1994). TS protein has been reported to interact with its own and other mRNAs (p53 and c-myc), inhibiting mRNA translation and leading to decreased specific protein levels (Van Triest et al., 2000; Schmitz et al., 2001). In addition, antisense nucleic acids targeted to the TS mRNA translation start site stimulate TS gene transcription (DeMoor et al., 1998), possibly by interfering with associations between that region of TS mRNA and cellular components that function to suppress TS
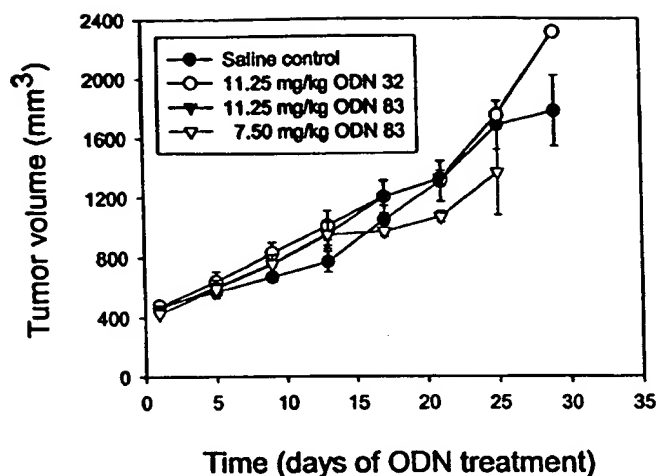
Fig. 4. Systemic administration of TS antisense ODN 83 has no effect on larger, established HT29 tumors in nude mice. Nude mice were injected subcutaneously with HT29 tumor cells, and tumors were allowed to grow to a size of 400 mm³ before treatment with ODNs as described for Fig. 1. Shown are the average tumor volume (± standard error) from five mice treated with saline (filled circles), 11.25 mg/kg control scrambled ODN 32 (open circles), 11.25 (filled triangles) or 7.5 (open triangles) mg of TS antisense ODN 83 per kg of body weight.

gene transcription. Studies are under way in our laboratory to dissect the molecular signaling pathways that lead to cell cycle arrest at G_2/M following TS antisense ODN treatment.

In contrast to apoptosis induced by direct inhibitors of TS protein (Van Triest et al., 2000), we found no increase in cell death in response to cell cycle arrest by TS antisense ODN in vitro. Considering that imbalances in dTTP/dUTP levels and DNA damage caused by inhibitors of TS enzyme activity can result in induction of downstream events leading to apoptosis, this indicates that antisense targeting has novel consequences that do not simply achieve the same results as TS protein targeting by a different route. The potential of treatment with antisense reagents to induce G_2/M arrest and inhibit cell proliferation without enhancing cell death, and the mechanism by which this occurs, is a previously undescribed but important area of future investigation.

In the in vivo experiments described here, mice bearing HT29 tumors were treated every 2nd day with ODN, resulting in decreased TS protein levels and reduced tumor size (by approximately 50%) after 4 weeks of growth. No overt toxicity (i.e., weight loss) was apparent in animals treated with TS antisense ODN 83. Thus, antisense strategies to target TS have the potential to be effective cytostatic methods to reduce human tumor growth. Although there was no obvious change in mitotic index associated with reduced growth, only tumors greater than 600 mm³ in volume were assessed microscopically to determine the metaphase fraction. In fact, tumors allowed to grow to 400 mm³ prior to ODN administration were unaffected by antisense TS. Together, these data suggest that treatment with TS antisense ODN in vivo may ultimately be of greatest value in adjuvant therapy of residual or nascent disease rather than in treatment of gross tumors.

Typical of rapidly growing adenocarcinomas, the large, well developed HT29 tumors had necrotic centers and appeared to be poorly vascularized. This might result in poor ODN delivery to the interior of large tumors, consistent with imaging f radiolabeled ODNs that preferentially localized at th periphery f tumors. The tumor periphery also contained the greatest proportion of cells whose appearance was consistent with higher viability and prolif rati n rate. Although dissociation of radiolabeled phosphate from ODN is theoretically possible and a potential complicating factor in interpretation, persistence of proliferating cells in the presence f high antisense ODN levels suggests that the antisense reagent has diminished capacity to inhibit cell proliferation in larger tumors, or that decreased TS levels might hav less influence on cell proliferation in macroscopic versus microscopic tumors. TS levels in tumor extracts were reduced by more than 40% by antisense TS treatment, and the possible presence of TS derived from mouse tissue or HT29 cells that failed to internalize ODN suggests that the observed reduction in TS protein underestimates the true effectiveness of antisense treatment. Although detailed examination of ODN distribution and effectiveness at the cellular level is required to resolve this issue, these data indicate a potential tum r size-dependent difference in response to TS antisense treatment.

The present results show clearly that systemic treatment with TS antisense ODN 83 as a single agent significantly d lays HT29 tumor growth in nude mice (Fig. 3), similar to the effect of raltitrexed alone (data not shown). We demonstrated previously that HeLa cells treated in vitro with TS antisense ODN 83 are sensitized to cytotoxicity of 5-FUdR or raltitrexed (Ferguson et al., 1999), and further studies are under way to test the hypothesis that sensitization occurs in vivo in microscopic and/or macroscopic tumors. Such a combination treatment may be necessary to overcome drug resistance mediated by TS upregulation in tumors in response to anti-TS chemotherapy (Gorlick and Bertino, 1999). We have observed in vitro that antisense ODN 83 effectively sensitized drug-selected, highly resistant HeLa cells to 5-FUdR cytotoxicity (P. J. Ferguson, in preparation), supporting this hypothesis. Antisense down-regulation of several specific mRNA targets sensitizes cells to chemotherapeutic drugs in vitro. For example, antisense to protein kinase Cα enhances sensitivity to mitomycin C, vincristine, and 5-FU (Chakrabarty and Huang, 1996), and antisense to c-myb sensitizes cells to cisplatin (Del Bufalo et al., 1996). The use of antisense ODNs to down-regulate Bcl-2 (Miayake et al., 2000) or tubulin (Kavallaris et al., 1999; Kyu-Ho Han et al., 2000) enhances paclitaxel sensitivity in drug-resistant tumor cells. This rational approach to combat tumor cell chemotherapy resistance, also demonstrated for TS in vitro (Ferguson et al., 1999; P. J. Ferguson, in preparation), is an important ongoing area of investigation.

In summary, an antisense ODN targeting TS was shown to be an effective single-agent antitumor therapy in vivo. Distribution of ODNs into solid tumors may be a limiting factor in efficacy against larger tumors. Further in vivo studies to examine the potency of combination therapies using this antisense ODN in combination with raltitrexed and 5-FU are in progress. In HeLa and HT29 cells in vitro, down-r gula-tion of TS mRNA and protein by treatment with TS antisense ODN 83 resulted in inhibition of proliferation, in the absence of increased cell death, via an immediate and sustained G_2/M cell cycle block. This was in contrast to the effect of TS-targeting chemotherapeutic drugs, which block cells in G_1/S and lead to increased apoptosis. Further studies are required to determine the molecular signaling pathways that mediate
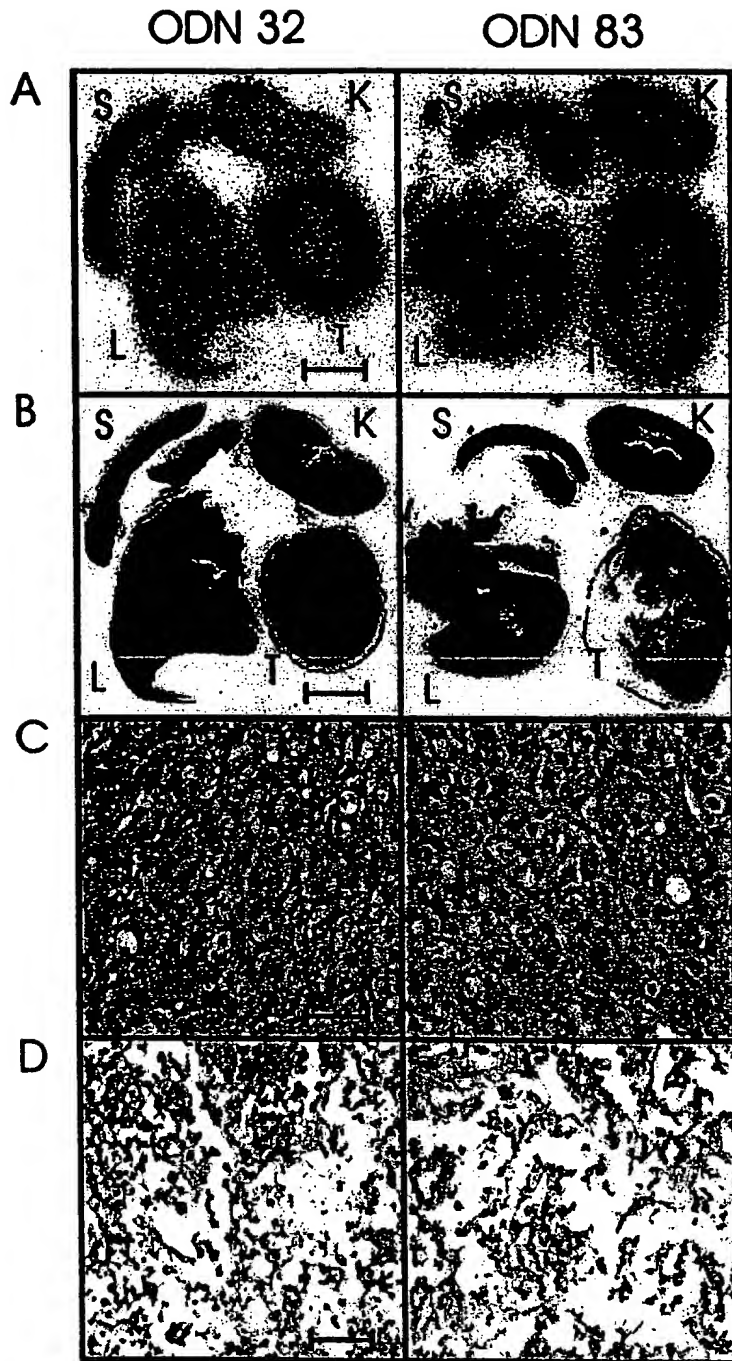
Fig. 5. Distribution of radiolabeled ODNs in HT29 tumors and normal mouse tissue. Mice bearing HT29 tumors that had been treated as described for Fig. 3B with control scrambled ODN 32 (left panels) or TS antisense ODN 83 (right panels) were injected with a trace amount of end-labeled ODN. A, radioactive decay images from sections of mouse spleen (S), kidney (K), liver (L), and HT29 tumors (T). B, hematoxylin and eosin staining of adjacent sections. Photomicrographs of the periphery (C) and the interior (D) of the tumors show cellular morphology. Scale bars, 5 mm (A and B) and 40 μm (C and D).

TABLE 2

TS protein levels in HT29 tumors in mice treated with ODNs

Tumor samples from mice treated with control scrambled ODN 32 or TS antisense ODN 83 were collected and analyzed for [6-^{3}H]5-FdUMP binding as described under *Materials and Methods*. Data shown are for tumors described in Fig. 3B.

Treatment	TS Levels[a]	% Reduction[b]
ODN 32	73.8 ± 8.6	
ODN 83	42.2 ± 13.9*	42.8

* Significant difference from ODN 32-treated mice (p = 0.003, Student's t test).
[a] [6-^{3}H]-FdUMP binding in tumor extracts, expressed as nmol/mg of protein (± S.E.M., n = 5).
[b] Reduction in TS level due to ODN 83 treatment, calculated as percentage of that in tumors from ODN 32-treated mice.

the G$_2$/M arrest and to ascertain how this phenomenon might be further exploited in novel antitumor therapies.

References

Agarwal ML, Agarwal A, Taylor WR and Stark GR (1995) p53 controls both the G2/M and the G1 cell cycle checkpoints and mediates reversible growth arrest in human fibroblasts. *Proc Natl Acad Sci USA* 92:8493–8497.

Agrawal S, Temsamani J and Tang JY (1991) Pharmacokinetics, biodistribution and stability of oligodeoxynucleotide phosphorothioates in mice. *Proc Natl Acad Sci USA* 88:7595–7599.

Chakrabarty S and Huang S (1996) Modulation of chemosensitivity in human colon carcinoma cells by downregulating protein kinase C alpha expression. *J Exp Ther Oncol* 1:218–221.

Chu E, Cogliati T, Copur SM, Borre A, Voeller DM, Allegra CJ and Segal S (1996) Identification of in vivo target RNA sequences bound by thymidylate synthase. *Nucleic Acids Res* 24:3222–3228.

Chu E, Koeller DM, Casey JL, Drake JC, Chabner BA, Elwood PC, Zinn S and Allegra CJ (1991) Autoregulation of human thymidylate synthase messenger RNA translation by thymidylate synthase. *Proc Natl Acad Sci USA* 88:8977–8981.

Chu E, Takechi T, Jones KL, Voeller DM, Copur SM, Maley GF, Maley F, Segal S and Allegra CJ (1995) Thymidylate synthase binds to c-myc RNA in human colon cancer cells and in vitro. *Mol Cell Biol* 15:179–185.

Chu E, Voeller DM, Jones KL, Takechi T, Maley GF, Maley F, Segal S and Allegra CJ (1994) Identification of a thymidylate synthase ribonucleoprotein complex in human colon cancer cells. *Mol Cell Biol* 14:207–213.

Danenberg PV, Malli H and Swenson S (1999) Thymidylate synthase inhibitors. *Semin Oncol* 26:621–631.

Dean N, McKay R, Miraglia L, Howard R, Cooper S, Giddings J, Nicklin P, Meister L, Ziel R, Geiger T, Muller M and Fabbro D (1996) Inhibition of growth of human tumor cell lines in nude mice by an antisense of oligonucleotide inhibitor of protein kinase C-alpha expression. *Cancer Res* 56:3499–3507.

Del Bufalo D, Cucco C, Leonetti C, Citro G, D'Agnano I, Benassi M, Geiser T, Zon G, Calabretta B and Zupi G (1996) Effect of cisplatin and c-myb antisense phosphorothioate oligodeoxynucleotides combination on a human colon carcinoma cell line in vitro and in vivo. *Br J Cancer* 74:387–393.

DeMoor JM, Vincent MD, Collins OM and Koropatnick J (1998) Antisense nucleic acids targeted to the thymidylate synthase (TS) mRNA translation start site stimulate TS gene transcription. *Exp Cell Res* 243:11–21.

El Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, Lin D, Mercer WE, Kinzler KW and Vogelstein B (1993) WAF1, a potential mediator of p53 tumor suppression. *Cell* 75:817–825.

Ferguson PJ, Collins O, Dean NM, DeMoor J, Li CS, Vincent MD and Koropatnick J (1999) Antisense down-regulation of thymidylate synthase to suppress growth and enhance cytotoxicity of 5-FUdR, 5-FU and Tomudex in HeLa cells. *Br J Pharmacol* 127:1777–1786.

Gorlick R and Bertino JR (1999) Drug resistance in colon cancer. *Semin Oncol* 26:606–611.

Hamada K, Zhang WW, Alemany R, Wolf J, Roth JA and Mitchell MF (1996) Growth inhibition of human cervical cancer cells with the recombinant adenovirus p53 in vitro. *Gynecol Oncol* 60:373–379.

Hasegawa S, Koshikawa N, Momiyama N, Moriyama K, Ichikawa Y, Ishikawa T, Mitsuhashi M, Shimada H and Miyazaki K (1998) Matrilysin-specific antisense oligonucleotide inhibits liver metastasis of human colon cancer cells in a nude mouse model. *Int J Cancer* 76:812–816.

Inaba M and Mitsuhashi J (1994) Flow cytometric analysis of cell-killing actions of 5-fluorouracil in human colorectal cancer cells. *Oncol Res* 6:303–309.

Johnson LF (1994) Posttranscriptional regulation of thymidylate synthase gene expression. *J Cell Biochem* 54:387–392.

Ju J, Pedersen-Lane J, Maley F and Chu E (1999) Regulation of p53 expression by thymidylate synthase. *Proc Natl Acad Sci USA* 96:3769–3774.

Kavallaris M, Burkhart CA and Horwitz SB (1999) Antisense oligonucleotides to class III beta-tubulin sensitize drug-resistant cells to Taxol. *Br J Cancer* 80:1020–1025.

Keyomarsi K, Samet J, Molnar G and Pardee AB (1993) The thymidylate synthase inhibitor, ICI D1694, overcomes translational detainment of the enzyme. *J Biol Chem* 268:15142–15149.

Kyu-Ho Han E, Gehrke L, Tahir SK, Credo RB, Cherian SP, Sham H, Rosenberg SH and Ng S (2000) Modulation of drug resistance by alpha-tubulin in paclitaxel-resistant human lung cancer cell lines. *Eur J Cancer* 36:1565–1571.

Leonetti C, D'Agnano I, Lozupone F, Valentini A, Geiser T, Zon G, Calabretta B, Citro GC and Zupi G (1996) Antitumor effect of c-myc antisense phosphorothioate oligodeoxynucleotides on human melanoma cells in vitro and in mice. *J Natl Cancer Inst* 88:419–429.

Matsui SI, Arredondo MA, Wrzosek C and Rustum YM (1996) DNA damage and p53 induction do not cause ZD1694-induced cell cycle arrest in human colon carcinoma cells. *Cancer Res* 56:4715–4723.

Miayake H, Tolcher A and Gleave ME (2000) Chemosensitization and delayed androgen-independent recurrence of prostate cancer with the use of antisense Bcl-2 oligodeoxynucleotides. *J Natl Cancer Inst* 92:34–41.

Munoz-Pinedo C, Oliver FJ and Lopez-Rivas A (2001) Apoptosis of haematopoietic cells upon thymidylate synthase inhibition is independent of p53 accumulation and CD95-CD95 ligand interaction. *Biochem J* 353 (Pt 1):101–108.

Navalgund LG, Rossana C, Muench AJ and Johnson LF (1980) Cell cycle regulation of thymidylate synthetase gene expression in cultured mouse fibroblasts. *J Biol Chem* 255:7386–7390.

Papamichael D (1999) The use of thymidylate synthase inhibitors in the treatment of advanced colorectal cancer: current status. *Oncologist* 4:478–487.

Rodrigues NR, Rowan A, Smith ME, Kerr IB, Bodmer WF, Gannon JV and Lane DP (1990) p53 mutations in colorectal cancer. *Proc Natl Acad Sci USA* 87:7555–7559.

Saijo Y, Perlaky L, Wang H and Busch H (1994) Pharmacokinetics, tissue distribution and stability of antisense oligodeoxynucleotide phosphorothioate ISIS 3466 in mice. *Oncol Res* 6:243–249.

Schmitz JC, Yu D, Agrawal S and Chu E (2001) Effect of 2'-O-methyl antisense ORNs on expression of thymidylate synthase in human colon cancer RKO cells. *Nucleic Acids Res* 29:415–422.

Shibata J, Aiba K, Shibata H, Minowa S and Horikoshi N (1998) Detection and quantitation of thymidylate synthase mRNA in human colon adenocarcinoma cell line resistant to 5-fluorouracil by competitive PCR. *Anticancer Res* 18:1457–1463.

Spears CP and Gustavsson BG (1988) Methods for thymidylate synthase pharmacodynamics: serial biopsy, free and total TS, FdUMP and dUMP, and H4PteGlu and CH2-H4PteGlu assays. *Adv Exp Med Biol* 244:97–106.

Stein CA and Cheng YC (1993) Antisense oligonucleotides as therapeutic agents—is the bullet really magical? *Science (Wash DC)* 261:1004–1012.

Stewart N, Hicks GG, Paraskevas F and Mowat M (1995) Evidence for a second cell cycle block at G2/M by p53. *Oncogene* 10:109–115.

Van Triest B, Pinedo HM, Giaccone G and Peters GJ (2000) Downstream molecular determinants of response to 5-fluorouracil and antifolate thymidylate synthase inhibitors. *Ann Oncol* 11:385–391.

Yin MB, Guo B, Panadero A, Frank C, Wrzosek C, Slocum HK and Rustum YM (1999) Cyclin E-cdk2 activation is associated with cell cycle arrest and inhibition of DNA replication induced by the thymidylate synthase inhibitor Tomudex. *Exp Cell Res* 247:189–199.

Address correspondence to: Dr. J. Koropatnick, Cancer Research Laboratories, The London Regional Cancer Center, 790 Commissioners Rd., London, ON, Canada, N6A 4L6. E-mail: jkoropat@uwo.ca